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Novel therapeutic targets in gastrointestinal stromal tumor

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ACADEMIC DISSERTATION

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ABBREVIATIONS

AIO	Arbeitsgemeinschaft Internistische Onkologie
AKT	protein kinase B
α4β1	very late antigen-4 (VLA-4)
α4β7	lymphocyte Peyer patch adhesion molecule
ANO1	anoctamin 1 (DOG1)
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
bHLH	basic helix-loop-helix
β-TrCP1	F-box/WD repeat-containing protein 1A
CALM2	calmodulin 2
CaM	calmodulin
CD117	Cluster of differentiation 117 (KIT)
CD29	Cluster of differentiation 29
CD49d	Cluster of differentiation 49d
cGMP	cyclic guanosine monophosphate
CLL	chronic lymphocytic leukemia
COPD	chronic obstructive pulmonary disease
CREB	cAMP response element-binding protein
DAB	3,3'-diaminobenzidine
DMSO	dimethyl sulfoxide
DNMDP	6-(4-(diethylamino)-3-nitrophenyl)-5-methyl-4,5-dihydropyridazin-3(2H)-one
DOG1	discovered on gastrointestinal stromal tumor 1 (ANO1)
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EIF4E	eukaryotic translation initiation factor 4E
EMT	epithelial-mesenchymal transition
EPAC	exchange factor directly activated by cAMP
FAK	focal adhesion kinase
FBS	fetal bovine serum
FDA	U.S. Food & Drug Administration
FFPE	formalin-fixed paraffin-embedded
FGF	fibroblast growth factor
FOXF1	forkhead box F1
G6PD	glucose-6-phosphate dehydrogenase
GIST	gastrointestinal stromal tumor
GPCR	G protein–coupled receptor
GSK-3β	glycogen synthase kinase-3β
GTI	gene tissue index
HDAC1/2	histone deacetylase 1/2
HeLa	HeLa cell line
HGF	hepatocyte growth factor
HTS	high-throughput screening
HPF	high-power field

HR	hazard ratio
IC50	half maximal inhibitory concentration
ICC	interstitial cells of Cajal
IHC	immunohistochemistry
ITGA4	integrin alpha 4
KIT	KIT proto-oncogene receptor tyrosine kinase
Ki67	Ki-67 antigen
LATS2	large tumor suppressor kinase 2
L1CAM	L1-cell adhesion molecule
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LNA	fluorescein-labelled locked nucleic acid
LSD1	lysine-specific demethylase 1
MAd-CAM-1	mucosal vascular addressin cell adhesion molecule-1
MAPK	mitogen-activated protein kinase
MET	MET proto-oncogene
miRNA	micro-RNA
MMP	matrix metalloproteinase
mRNA	messenger-RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NES	nuclear export sequence
NF1	neurofibromatosis type 1
NF-κB	nuclear factor-κB
NIH	U.S. National Institutes of Health
NMRI	Naval Medical Research Institute
PARP	poly (ADP-ribose) polymerase
PDGFRA	platelet-derived growth factor receptor α
PDE	cyclic nucleotide phosphodiesterase
PDE3A	phosphodiesterase 3A
PDE3B	phosphodiesterase 3B
pHH3	phosphohistone H3
PAS	Per-Arnt-Sim
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PKD1	polycystin 1
PI3K	phosphoinositide 3-kinase
PPP1CB	protein phosphatase 1 catalytic subunit beta
PRC2	polycomb repressive complex 2
PRKCQ	protein kinase c theta
PRMT5	protein arginine methyltransferase 5
PROTAC	proteolysis targeting chimeras
qPCR	quantitative PCR
RAD23	nucleotide excision factor protein
RIPA	radioimmunoprecipitation assay buffer
RFS	recurrence-free survival
RGS	regulators of G protein signaling
RTK	receptor tyrosine kinase

SCP	small C-terminal domain phosphatase
SDH	succinate dehydrogenase
SePIA	sequence processing, integration, and analysis
SFC	stem cell factor
SFK	SRC family kinase
SLFN12	schlafen family member 12
SLUG	snai2 protein
SNAI1	snail family transcriptional repressor 1
SNAI2	snail family transcriptional repressor 2
siRNA	small interfering RNA
SRD	serine rich domain
SSG	Scandinavian Sarcoma Group
Suv39H1	suppressor of variegation 3-9 homolog 1
TBP	TATA-binding protein
TNF-α	tumor necrosis factor-α
TGF-β	transforming growth factor-β
TMA	tissue microarray
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
URC	uracil catabolism protein
VCAM-1	vascular cell adhesion molecule-1
YWHAQ	14-3-3 protein theta

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by Roman numerals (I-III).

I Pulkka OP, Nilsson B, Sarlomo-Rikala M, Reichardt P, Eriksson M, Hall KS, Wardelmann E, Vehtari A, Joensuu H, and Sihto H. SLUG transcription factor: a pro-survival and prognostic factor in gastrointestinal stromal tumour. Br J Cancer. 116: 1195–1202, 2017.

II Pulkka OP, Mpindi JP, Tynninen O, Nilsson B, Kallioniemi O, Sihto H, and Joensuu H. Clinical relevance of integrin alpha 4 in gastrointestinal stromal tumours. J Cell Mol Med. 22: 2220-2230, 2018.

III Pulkka OP, Gebreyohannes YK, Wozniak A, Mpindi JP, Tynninen O, Icay K, Cervera A, Keskitalo S, Murumägi A, Kulesskiy E, Wennerberg K, Varjosalo M, Laakkonen P, Lehtonen R, Hautaniemi S, Kallioniemi O, Schöffski P, Sihto H, Joensuu H. Anagrelide for Gastrointestinal Stromal Tumor. Clin Cancer Res. 25:1676-1687, 2019.

ABSTRACT

Background: Gastrointestinal stromal tumor (GIST) is one of the most common types of soft tissue sarcoma. The molecular mechanisms of GISTs are incompletely understood though the importance of KIT or platelet-derived growth factor α (PDGFRA) signaling in GIST is evident. The molecular mechanisms beyond KIT and PDGFRA signaling are incompletely understood. Tyrosine kinase inhibitors and especially imatinib, an inhibitor of KIT, PDGFRA and BCL-ABL, revolutionized the systemic treatment of GIST. However, advanced GISTs usually eventually progress on tyrosine kinase inhibitors, often because of secondary *KIT* mutations. There is a need for novel effective agents for the treatment of patients with GIST.

Experimental design: The GIST gene expression profile was investigated in an *in silico* transcriptome database comprising of human tissue and cancer samples. Two GIST cell lines were screened for sensitivity to 217 anti-cancer compounds. SLUG, ITGA4, PDE3A and PDE3B expression was studied using immunohistochemistry on tissue microarrays (TMA). We used three different clinical cancer patient series: the first was a series that consisted of samples from 630 tumors from the archives of the Department of Pathology, Helsinki University Hospital; the second was a population-based cohort consisting of GIST patients who were treated with surgery in Western Sweden from 1983 through 2000; and the third series consisted of high risk GIST patients who were entered to the Scandinavian Sarcoma Group (SSG) XVIII/Arbeitsgemeinschaft Internistische Onkologie (AIO) adjuvant trial. The effects of SLUG, ITGA4 and PDE3 knockdown and selective ITGA4 and PDE3 inhibitors were investigated in three GIST cells lines. The efficacy of a PDE3 inhibitor, anagrelide, was investigated in patient-derived xenograft mouse models.

Results: SLUG was expressed in 25.0 %, ITGA4 in 52.3 %, PDE3A in 90.9 % and PDE3B in 60.0 % of the GISTs investigated. Expression of these proteins were also detected in some other human tumor types, but usually much less frequently. SLUG and ITGA4 expression were associated several factors linked with unfavorable prognosis. SLUG expression was associated significantly also with unfavorable recurrence-free survival both when the patients were treated with surgery alone and when treated with surgery followed by adjuvant imatinib. ITGA4 expression was associated with unfavorable GIST-specific survival and overall survival in a patient population treated with surgery alone. PDE3A and PDE3B expression had no significant associations with the clinicopathological factors studied, RFS or overall survival. SLUG and PDE3 downregulation inhibited cell proliferation

and induced apoptosis in GIST cell lines, whereas ITGA4 inhibition decreased GIST cell invasion. Anagrelide reduced or stabilized tumor growth in several GIST xenograft mouse models.

Conclusions: SLUG, ITGA4 and PDE3s are frequently expressed in GISTs. They are likely important factors in the molecular pathogenesis of GIST, and may influence their clinical behavior. As ITGA4 and PDE3s can be targeted with specific inhibitors, they could potentially be therapeutic targets in GIST. SLUG may mediate pro-survival signaling in GIST. Some PDE3 inhibitors, such as anagrelide, warrant more study as potential therapeutic agents in GIST

INTRODUCTION

Gastrointestinal stromal tumor (GIST) is one the most common types of sarcoma, affecting 10 to 15 people per million per year (Soreide et al. 2016). GIST can arise anywhere along the gastrointestinal tract, but is found most often in the stomach or the small bowel (Guller et al. 2015). GISTs respond poorly to standard cytotoxic chemotherapy and were a particularly challenging type of cancer to treat until the year 2000, when the first patient with metastatic GIST was treated with imatinib with a dramatic response (Joensuu et al. 2001).

Approximately 75% of GISTs harbor an activating mutation of the KIT receptor tyrosine kinase, and one third of GISTs that lack *KIT* mutation contain a mutation in the *platelet-derived growth factor receptor α* (*PDGFRA*) gene (Heinrich et al. 2003, Joensuu et al. 2015). Imatinib mesylate that targets KIT and PDGFRA is the established adjuvant therapy for patients whose tumor is considered to have a high risk for recurrence after surgery, and the standard first-line therapy for patients with metastatic GIST (Demetri et al. 2002, Joensuu et al. 2016). However, drug resistance frequently emerges when patients with advanced GIST are treated with tyrosine kinase inhibitors (Heinrich et al. 2006). Although *KIT* and *PDGFRA* mutations are likely of key importance in the molecular pathogenesis of GISTs, identical single *KIT* or *PDGFRA* mutations are often associated with widely different GIST patient survival outcomes and tumor mitotic counts (Joensuu et al. 2015). Therefore, aberrations in several other genes may be crucially important in determining the function of GIST cells. For example, the transcription factor ETV1 expression has demonstrated to cooperate with KIT activation and the forkhead family member FOXF1 directly controls the transcription of both *ETV1* and *KIT* (Chi et al. 2010, Ran et al. 2017).

Despite the great success of imatinib in the treatment of GIST, several other kinase inhibitors have been associated with only modest further clinical benefit in the metastatic setting. Ongoing clinical trials are focused on tyrosine kinase inhibitors, and there is a lack of molecules that could be novel targets for GIST therapy.

The aim of the present study was to investigate the clinical significance of the zinc finger protein SNAI2 (SLUG), integrin alpha 4 (ITGA4) and phosphodiesterase 3A and 3B (PDE3A and PDE3B) in GIST. In addition, we wanted to investigate the effects of inhibition of PDE3 on GIST cell lines

and xenografts, and to study whether these proteins could be a potential therapeutic target in the treatment of GIST.

REVIEW OF THE LITERATURE

1. Gastrointestinal stromal tumor (GIST)

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract. These tumors were formerly classified as leiomyomas, leiomyosarcomas or leiomyoblastomas, but are now recognized as a separate tumor entity (Fletcher et al. 2002). The term "stromal tumor" was first introduced in 1983, when Mazur and Clark discovered neoplasms that lacked the immunophenotypic features of smooth-muscle differentiation or Schwann cells (Mazur and Clark. 1983). In the mid-1990s, researchers noted that there are clear similarities between GIST cells and the interstitial cells of Cajal, which serve as pacemakers for the peristaltic contractions of the gastrointestinal tract (Huizinga et al. 1995, Kindblom et al. 1998, Sircar et al. 1999). Both types of cells express the KIT receptor tyrosine kinase and are dependent on the stem cell factor (Huizinga et al. 1995). A groundbreaking finding was made in 1998 when Hirota and his colleagues reported that gain-of function mutations in the *KIT* receptor tyrosine kinase (RTK) gene are present in the majority of GISTs (Hirota et al. 1998).

GISTs were considered generally resistant to chemotherapy, and patients with GIST had often poor outcome. The new insights of the biological character of GIST, together with the introduction of tyrosine kinase inhibitor (TKI) treatments, increased the interest in GIST and revolutionized the treatment of this tumor type (Druker et al. 2001, Joensuu et al. 2001). In 2003, activating mutations in the platelet derived growth factor receptor alpha (*PDGFRA*) gene, which belongs to same family of receptor tyrosine kinases as KIT, were discovered in some GISTs (Heinrich et al. 2003, Hirota et al. 2003). Approximately 85 % of GISTs harbor an activating mutation in either *KIT* or *PDGFRA* (Corless et al. 2004, Joensuu et al. 2015). The rest of GISTs (10-15 %) may harbor mutations in other genes, including *SDH* (succinate dehydrogenase), *NF1* (neurofibromatosis type 1), *BRAF* or *KRAS* (Pantaleo et al. 2015).

Inhibition of KIT and PDGFRA oncoproteins with tyrosine kinase inhibitors made inoperable and metastatic GISTs treatable, and made GIST a model of effective targeted therapies among solid cancers. Despite the success of tyrosine kinase inhibitors, drug resistance frequently emerges in these tumors, and new therapy approaches are thus needed.

1.1 Epidemiology

The incidence of GIST around the world is already quite well determined, even though GISTs have been properly recognized and diagnosed only since the late 1990s. A recent systematic review of the global epidemiology of GIST that included altogether 29 studies comprising more than 13,550 patients from 19 countries found the reported annual incidence of GIST varies between 4.3 and 22.0 cases/million/year (Soreide et al. 2016). However, most of the studies report an incidence between 10 and 15 cases per million. The studies found an increased risk and a higher incidence with advancing age with age-related incidence over 30 per million for patients over 70 years of age (Ma et al. 2015, Yan et al. 2008). The annual incidence is approximately 7,500-11,000 new cases in Europe and approximately 3,000-5,000 cases in the USA.

GISTs are thus generally found in older people, and the median age at diagnosis is in the mid-60s in most of the population-based studies. GIST are rarely found in children and young adults (Miettinen et al. 2005, Prakash et al. 2005). GISTs have no clear gender distribution (Miettinen et al. 2005), although in most series GISTs are slightly more common in males than in females. A few families with a germ-line *KIT* or *PDGFRA* mutation exist, but GISTs are usually sporadic (Li et al. 2005, Nishida et al. 1998). In addition, a few syndromes, neurofibromatosis type I, the Carney's triad (GIST, extra-adrenal paraganglioma, and pulmonary chondroma without SDH-mutation), and the Carney-Stratakis syndrome (GIST, extra-adrenal paraganglioma, and pulmonary chondroma with SDH-mutation) are associated with frequent GIST development (Carney. 1999, Miettinen et al. 2006, Pasini et al. 2008).

1.2 Clinical features

GISTs can exist anywhere along the gastrointestinal tract but are found most often in the stomach (~55%), small intestine (~32%) or rectum (~6%), and only rarely in other locations (Soreide et al. 2016). The majority of the GIST patients have local disease, but about 20% of GISTs are metastatic at the time of the diagnosis (Guller et al. 2015). Metastases occur usually in the liver or other intra-abdominal sites, whereas metastases outside of the abdomen are uncommon (Dematteo et al. 2002, Nilsson et al. 2005, Zaydfudim et al. 2012). Metastases may occur up to 20 years after initial surgery (Joensuu et al. 2012). Clinical symptoms such as abdominal pain, bleeding, anemia, fatigue,

dysphagia, satiety and obstruction are associated with GIST (Nilsson et al. 2005). Patients may also present a tumor with no symptoms.

The median tumor size is approximately 7 cm at the time of GIST diagnosis (Guller et al. 2015). Almost 50 % of tumors are larger than 5 cm, and only 13 % in the < 2 cm size group (Soreide et al. 2016). GISTs were first grouped to four NIH risk categories (very low, low, intermediate and high) based on tumor size and mitotic counts (Fletcher et al. 2002). Newer risk-stratification schemes for operable GIST are also available, based on tumor mitotic count, site, size, and presence of tumor rupture (Gold et al. 2009, Joensuu. 2008, Miettinen and Lasota. 2006, Rutkowski et al. 2011, Woodall et al. 2009). The novel prognostic heat maps and contour maps may, however, be more accurate than these risk-stratification schemes (Joensuu et al. 2012).

1.3 Histopathology

GISTs are usually well-circumscribed, highly vascular tumors that are surrounded by a pseudocapsule. The radiologic and gross appearance of GISTs can, however, be highly variable. GISTs may have intraluminal, intramural, and external components, pedunculated extramural components, and cystic appearances (Miettinen and Lasota. 2013). GISTs have three principal cytomorphologic subtypes: 1) the spindle-shaped type (~70%) displays cells with palely eosinophilic fibrillary cytoplasm, ovoid nuclei, and ill-defined cell borders; 2) the epithelioid type (~20%) composed of round cells with eosinophilic to clear cytoplasm arranged in sheets and nests, and 3) the mixed type (~10%) (Fletcher et al. 2002). Each subtype can have variable cellularity as well as sclerotic, collagenous, or myxoid stromal changes. Pleomorphic and dedifferentiated GISTs are also seen occasionally. Tyrosine kinase inhibitor treatments may induce dramatic changes in cellularity, and cause marked hyaline degeneration and other stromal alterations (Pauwels et al. 2005).

Immunohistochemical stainings are used to confirm the diagnosis of GIST. Most of the GISTs (~95%) stain positively for the KIT protein, and KIT is considered to a useful biomarker of GIST in the differential diagnosis (Nilsson et al. 2005, Sarlomo-Rikala et al. 1998). In most cases the staining is strong and diffuse, but the staining pattern can, however, vary from membranous and clearly cytoplasmic to a perinuclear dot-like pattern (Miettinen and Lasota. 2013). Approximately 5% of GISTs are KIT-negative (Debiec-Rychter et al. 2004). Another very useful GIST biomarker, which is frequently expressed also in KIT-negative GISTs, is anoctamin-1 (ANO1), also known as

"discovered on GIST" (DOG1) (West et al. 2004). ANO1 is a calcium-activated chloride channel protein expressed also in the Cajal cells (Hwang et al. 2009). At least 95 % of GISTs stain positively for ANO1 (Miettinen et al. 2009). Together KIT and ANO1 immunostains cover nearly 100 % of GISTs. Other commonly expressed, but less-specific and sensitive GIST markers include CD34, expressed in approximately 70 % of GISTs, and protein kinase C (PKC)-theta, expressed in about 85 % of GISTs (Miettinen et al. 2005, Motegi et al. 2005).

1.4 Molecular biology

Oncogenic *KIT* and *PDGFRA* mutations are the main molecular drivers of GIST. Both genes map to chromosome 4q12 and encode a type III receptor tyrosine kinase (Stenman et al. 1989). These structurally related kinases contain an extracellular ligand binding region, a transmembrane section, a juxtamembrane domain, and cytoplasmic kinase domains, one with an ATP-binding pocket and the other with a kinase activation loop (Figure 1) (Hubbard. 2004, Ullrich and Schlessinger. 1990). Normally KIT and PDGFRA are activated by binding of the stem cell factor and platelet derived growth factors, respectively. Binding of the ligands results to dimerization and cross-phosphorylation of the cytoplasmic kinase part that leads to activation of signaling routes regulating cell proliferation and survival (Blume-Jensen et al. 1991). KIT is also known to have a critical function in the development and maintenance of various cell types including the mast cell, melanocytes, hematopoietic cells, germ cells and the interstitial cells of Cajal (Blume-Jensen et al. 1991, Lev et al. 1993, Maeda et al. 1992). PDGFRA has a role in gastrulation and in the development of the cranial and cardiac neural crest, the lungs, the skin, the gonads, the intestine, the central nervous system, and the skeleton (Andrae et al. 2008)

KIT and *PDGFRA* mutations can be found in any of the parts of the genes that encode the protein domains, but they are most often found in *KIT* exon 11 that encodes for the juxtamembrane domain of the KIT protein (~90 % of *KIT* mutations and ~65% of all GISTs). Approximately 10 % of GISTs have exon 9 *KIT* mutation, encoding for the extracellular dimerization domain. Primary *KIT* mutations occur rarely (~2 %) in either exon 13 (encodes for the ATP binding pocket) or exon 17 (kinase activation loop) (Corless et al. 2004, Heinrich et al. 2003). Constitutively activating *PDGFRA* mutations are identified in approximately 10-15 % of GISTs. Unlike *KIT*, these mutations are usually found in the part of the gene that encodes the kinase activating loop (exon 18) or the ATP-binding

pocket (exon 14), and only relatively rarely in *PDGFRA* exon 12 that encodes the juxtamembrane domain (Joensuu et al. 2015).

KIT exon 11 mutated GISTs can occur anywhere along the gastrointestinal tract. Exon 11 mutations include missense mutations, insertions, deletions and infrequently internal tandem duplications (Fletcher and Rubin. 2007, Lasota and Miettinen. 2008). Tandem repeat mutations are associated with more favorable clinical outcome, whereas deletions are associated with a shorter progression-free and overall survival. Especially *KIT* exon 11 codon 557–558 deletions are associated with poor prognosis (Corless et al. 2011, Martin et al. 2005, Wardelmann et al. 2003), unless the patient is treated with adjuvant imatinib, which abolishes the adverse influence of this mutation on prognosis (Joensuu et al. 2017).

KIT exon 9 mutations occur most commonly in small intestinal GISTs that are frequently considered high-risk tumors (Antonescu et al. 2003, Miettinen et al. 2006). Most exon 9 mutations are characterized by a six base pair insertion introducing a tandem alanine-tyrosine pair (AY502-503) (Lasota and Miettinen. 2008). The kinase domain of exon 9 mutant KIT is similar to the wild type KIT, which affects imatinib efficacy, and patients with *KIT* exon 9 mutation are recommended to be treated with a high daily dose of imatinib (Debiec-Rychter et al. 2006, Marrari et al. 2010). *KIT* exon 13 and exon 17 mutations are rare in patients who have not been treated with tyrosine kinase inhibitors, and occur slightly more often in the small intestine GISTs as compared to other locations. Mutations in exon 13 and exon 17 are usually substitutions and occur often in codons 642 and 822, respectively (Lasota et al. 2008).

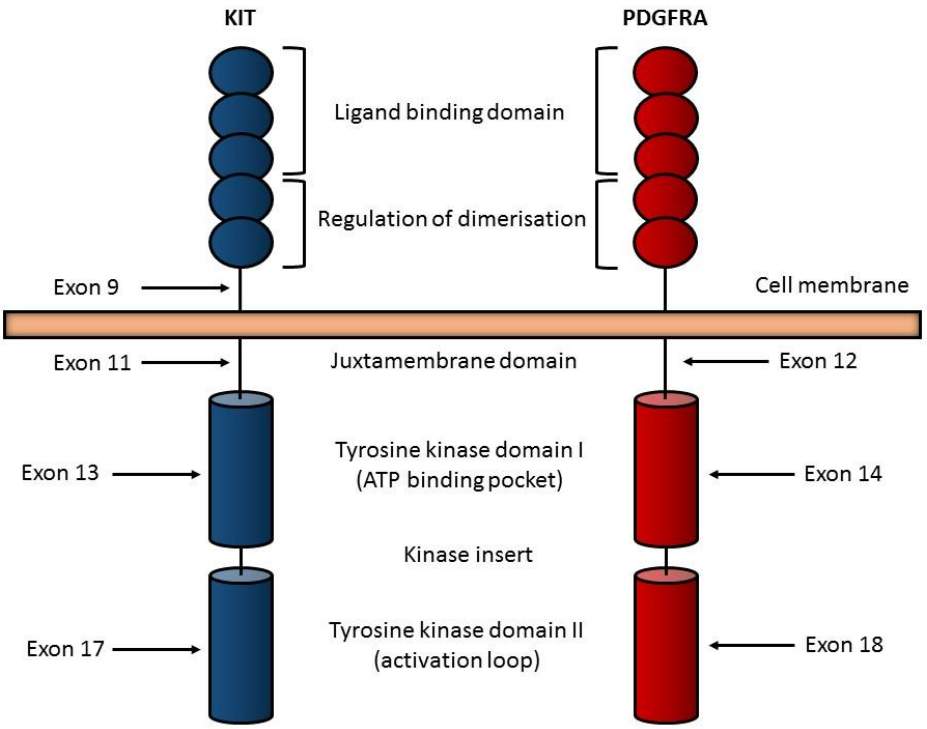

Figure 1. Structure of KIT and PDGFRA receptor tyrosine kinases. Adapted from Joensuu *et al*. 2013.

Most of the *PDGFRA* exon 14 and exon 18 mutations are missense mutations, but include also in-frame deletions and internal tandem duplications. Substitutions are most often found at D842 in exon 18, and D842V is notorious for its resistance to imatinib and sunitinib (Corless et al. 2005, Hirota et al. 2003). The majority of GISTs with a *PDGFRA* mutation arise in the stomach, and they usually have a favorable clinical course following surgery alone (Joensuu et al. 2015).

Up to about 10 % of GISTs do not have a detectable *KIT* or *PDGFRA* mutation. This GIST population was previously referred to as "wild-type" GIST, but is better named as "non-*KIT*, non-*PDGFRA*-mutated" GIST, since mutations in other genes may occur in this subset of GISTs. Recent studies have revealed that this is a heterogeneous group of GISTs with various oncogenic mutations or gene silencing. *BRAF* exon 15 V600E mutations are identified in some "wild-type" GISTs (Agaimy et al. 2009, Agaram et al. 2008, Hostein et al. 2010). Defects in the four genes (mutations or epigenetic changes) encoding for the succinate dehydrogenase (SDH) complex have been identified in the majority of "wild-type" GISTs (Boikos et al. 2016). SDH-deficient GISTs include especially pediatric

GISTs. Germline *SDH* mutations are associated with the development the Carney–Stratakis syndrome (Janeway et al. 2011). Patients with neurofibromatosis-1 have a high risk to develop a GIST. Most of such GISTs do not contain a *KIT* mutation, are located in the small-intestine and generally have favorable prognosis (Andersson et al. 2005, Miettinen et al. 2006).

1.5 Management of localized GIST

1.5.1. Surgery

Macroscopically complete (R0/R1) surgery is the standard treatment for localized GIST. GIST larger than 2 cm should be resected as well as smaller symptomatic (bleeding) GISTs (Miettinen and Lasota. 2006). Small to medium sized gastric GISTs are often treated with wedge resection and localized intestinal GISTs with segmental resection. Small and medium-sized GISTs are now increasingly often treated with laparoscopic surgery (Novitsky et al. 2006, Otani et al. 2006, Piessen et al. 2015). Larger GISTs require open surgery and more extensive resections. The aim of surgery is to achieve negative microscopic margins (R0). The optimal width of the tumor-free margin has not been defined, but there is little evidence suggesting that patients with microscopically positive margins (R1) require re-extension (McCarter et al. 2012). Tumor rupture during surgery should be avoided, as tumor rupture is associated with an increase of peritoneal implants and recurrence of the disease (Rutkowski and Ruka 2009). GIST do not usually give rise to lymph node metastases (apart from pediatric GISTs), and lymphadenectomy is not required.

1.5.2. Neoadjuvant (preoperative) imatinib treatment

Preoperative imatinib therapy may be given to selected patients with locally advanced GIST. Preoperative imatinib should be considered to shrink the tumor and allow organ sparing when up-front surgery might lead to extensive organ resections. Neoadjuvant treatment may be beneficial for patients with a large or unresectable tumor or when a small GIST is difficultly positioned, notably rectal GISTs (Rutkowski et al. 2013). The optimal duration of preoperative imatinib is unknown, but it is often administered for 6 to 12 months to allow maximal tumor shrinkage. Imatinib is continued until surgery and should be resumed after surgery to complete 3 years of treatment when the risk of recurrence is substantial. Neoadjuvant imatinib is not recommended for all patients, since not all GISTs respond to imatinib, and estimation of the need for adjuvant therapy may be challenging or

not possible after neoadjuvant imatinib treatment. GIST mutation status should be evaluated routinely prior to starting tyrosine kinase treatment for GIST.

1.5.3. Adjuvant (post-operative) imatinib treatment

Imatinib is the only tyrosine kinase inhibitor that has been studied in adjuvant treatment setting of operable GIST. In two randomized trials, adjuvant imatinib administered for either 1 year or 2 years improved recurrence-free survival but overall survival benefit was not observed (Casali et al. 2015, Corless et al. 2014). In the third trial, adjuvant imatinib was found to prolong overall survival in high risk patients. In the Scandinavian Sarcoma Group SSGXVIII trial that compared 3 years of adjuvant imatinib to 1 year of imatinib in a patient population considered to have a high risk of GIST recurrence after surgery alone the patients treated with imatinib for 3 years survived longer (Joensuu et al. 2012, Joensuu et al. 2016). Based on the results of this trial, both the European Society for Medical Oncology (ESMO) and the U.S. National Comprehensive Cancer Network (NCCN) now recommend at least 3 years of adjuvant imatinib for patients who have a high risk for GIST recurrence despite macroscopically radical surgery (ESMO/European Sarcoma Network Working Group. 2014). Two randomized trials are currently evaluating a longer than the 3-year duration of imatinib as the adjuvant treatment of high-risk GIST.

1.6 Management of advanced GIST and imatinib resistance

Imatinib is the standard first-line treatment for the patients with unresectable or metastatic GIST. Before the imatinib era, treatment of the patients with advanced GIST was ineffective. Only a minority of GIST patients (<10 %) responded to conventional chemotherapy, and the median survival time for the patients with advanced disease was only about 18 months (Dematteo et al. 2002). Imatinib treatment improved tremendously progression-free survival and overall survival of GIST patients in multiple studies. Imatinib was originally introduced for the treatment of chronic myeloid leukemia (CML) due to its ability to inhibit the fusion oncoprotein BCR-ABL (Druker et al. 1996). ABL has structural similarity with KIT and several other tyrosine kinases. Imatinib binds directly to the ATP binding pocket of KIT inhibiting the ATP binding (Mol et al. 2004).

Imatinib was first used clinically to treat a patient with metastatic GIST, and a dramatic response was seen (Joensuu et al. 2001). In the following international clinical trials 70-85 % of patients with

advanced GIST achieved disease control and the median time to disease progression was 18-24 months (Blanke et al. 2008, Demetri et al. 2002). Currently the median survival of the imatinib-treated patients with advanced disease is at least 5 years, with 19-23 % or more of the patients surviving more than 10 years (Blanke et al. 2008, Casali et al. 2017, Heinrich et al. 2017). Of the patients with oligometastatic (a small number of new tumors) GIST treated with metastasis surgery and prolonged imatinib therapy more than 50% now become 10-year survivors, and at present a multicenter clinical trial is evaluation whether some of these patients are cured from overtly metastatic GIST (the STOP GIST trial) (Hompland et al. 2017).

KIT and *PDGFRA* mutation status significantly predicts for the response to imatinib therapy. Imatinib resistance appearing within the first 6 months of imatinib treatment is referred to as primary resistance (Heinrich et al. 2003). Most of the patients usually achieve a good initial response or stable disease, but will develop tumor progression after a median of 2-3 years, referred as secondary resistance (Casali et al. 2017). Secondary resistance is usually caused by secondary *KIT* or rarely *PDGFRA* mutations in the tyrosine kinase domains (Debiec-Rychter et al. 2005). Secondary mutations in *KIT* are often found in exons 13, 14, 17 or 18 (Gajiwala et al. 2009, Joensuu et al. 2015).

Most of the GIST *KIT* exon 11 mutants are sensitive to imatinib, although some variants may show varying resistance (Lasota and Miettinen. 2008). Patients with *KIT* exon 11 mutation have the best response rates to imatinib. The median time to tumor progression is more than 1 year longer compared to *KIT* exon 9 mutants or other genomic subtypes (Debiec-Rychter et al. 2006, Heinrich et al. 2003). Clinical objective response rates (complete or partial response) for *KIT* exon 11 mutant GISTs, *KIT* exon 9 mutant GISTs, and "wild-type" GISTs are 64-84 %, 35-48 %, and 0-37 %, respectively (Heinrich et al. 2003, Martin et al. 2005, Wozniak et al. 2014). *PDGFRA* mutant GISTs may also respond to imatinib, but *PDGFRA* D842V is considered imatinib-resistant. The objective response rate for *PDGFRA* exon 12 mutant GIST and *PDGFRA* exon 18 mutant GIST are 30-100 % and 0-25 %, respectively (Heinrich et al. 2003, Martin et al. 2005, Wozniak et al. 2014).

The dose for imatinib is 400 mg per day, except for patients with *KIT* exon 9 mutant patients, who are recommended to be treated with 800 mg/day. About a third of the patients who progress on the 400 mg dose of imatinib benefit from dose escalation to 800 mg/day (Zalcberg et al. 2005). However, the higher imatinib dose is associated with greater toxicity, and the benefit usually lasts only for a few months. Patients with GIST with *KIT* exon 9 mutation have longer progression-free survival

when treated with 800 mg/day as compared with the 400 mg/day dose (Gastrointestinal Stromal Tumor Meta-Analysis Group (MetaGIST). 2010).

Although the majority of GIST patients with unresectable or metastatic GIST have durable response to imatinib, eventually most of the GISTs become resistant to imatinib. Secondary single nucleotide substitution mutations in *KIT* that encodes either the ATP binding pocket (exon 13 or exon 14) or the activation loop (exon 17) are the main cause for imatinib resistance (Heinrich et al. 2006, Wardelmann et al. 2006). The currently approved second and third line therapy options, sunitinib and regorafenib, have been found to effective in randomized trials in the treatment of GIST that has become resistant to imatinib, but the responses are generally shorter than to first-line imatinib (Demetri et al. 2006, Demetri et al. 2013).

Sunitinib is effective especially against secondary mutations located in the ATP binding pocket (Heinrich et al. 2008). In a randomized study sunitinib-treated patients had a longer time to tumor progression as compared with placebo (27.3 vs 6.4 weeks), and longer survival (Demetri et al. 2006). In the third-line setting in a patient population whose GIST had progressed on imatinib and sunitinib, patients treated with regorafenib had longer progression-free survival as compared with placebo (median progression-free survival 4.8 vs 0.9 months) (Demetri et al. 2013).

2. Phosphodiesterases

Cyclic nucleotide phosphodiesterases (PDEs) regulate the intracellular concentrations of the cyclic AMP (cAMP) and cyclic GMP (cGMP) by catalyzing their hydrolysis (Beavo 1995). The cAMP and cGMP signaling systems regulate various biological processes in health and disease. They regulate cell proliferation and differentiation, inflammation, apoptosis, gene expression and metabolic signaling. Properties of different receptors, G proteins, cyclases and protein kinases that regulate the synthesis of cAMP and cGMP are well established (Conti and Beavo 2007). Eleven PDE families have been identified in mammalian tissues. Each of these families typically has several different isoforms and splice variants (Bender and Beavo 2006). These variants are unique in tissue-expression, gene regulation, subcellular localization and interaction with other proteins.

PDEs have been recognized as promising drug targets in various diseases. Regulation of the degradation of a ligand or a second messenger often causes faster and more widespread changes in

the intracellular concentrations of proteins as compared to regulation the rate of their synthesis, which makes PDEs interesting therapeutic targets. Effective PDE-based therapies are available or in the development for a wide range of clinical applications, including inflammatory diseases, heart failure, erectile dysfunction, asthma and arrhythmias (Maurice et al. 2014).

2.1 Structure and function

The PDEs share a similar structural organization, but have different amino-terminal regulatory regions, and a conserved carboxy-terminal catalytic core (Figure 2). Of the twelve PDE families PDE4, PDE7 and PDE8 hydrolyze only cAMP, whereas PDE5, PDE6 and PDE9 hydrolyze cGMP, and PDE1, PDE2, PDE3, PDE10 and PDE11 hydrolyze both (Conti and Beavo 2007, Francis et al. 2011). The regulatory regions located in PDE N-terminal region retain structural factors that direct individual PDEs to various subcellular locations (Beavo 1995). The N-terminal region contains binding sites for ligands and allosteric effectors, dimerization domains, autoinhibitory modules, domains for isoform-specific protein–protein interactions and phosphorylation sites, and other covalent modification sites (Conti and Beavo 2007). Many cells express more than only one PDE family, but often in variable subcellular locations and proportions.

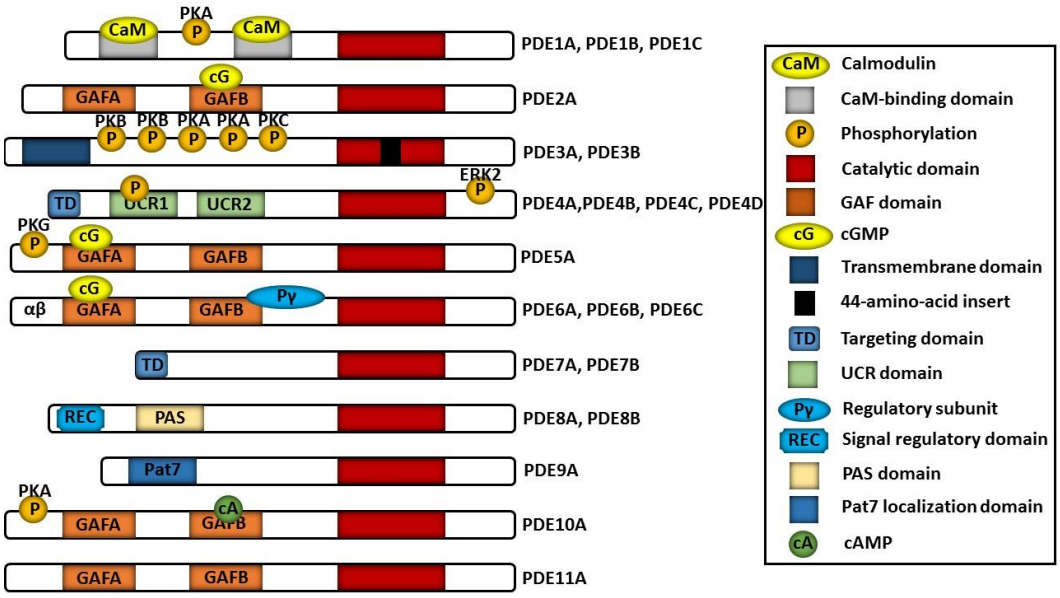

Figure 2. Structure and domain organization of the mammalian phosphodiesterase families. Adapted and modified from Maurice *et al*. 2014.

The catalytic domains of PDEs share a similar topography that is composed of ~350 amino acids folded into 16 helicases (Ke and Wang. 2007). These domains contain a PDE specific, histidine containing motif (HD(X_2) H(X_4)N), and binding sites for two divalent metal ions essential for catalytic functions (Ke et al. 2011). Conserved catalytic domains are responsible for binding to cAMP, and cGMP and some phosphodiesterase inhibitors. The catalytic core contains variable elements regulating PDE-specific substrate and inhibitor affinity and selectivity (Keravis and Lugnier 2012).

Various intracellular cyclic nucleotide targets highlight also the role of PDEs in controlling of cellular events. Cells regulate the intracellular levels of cAMP and cGMP in a highly categorized manner and generate separate intracellular cyclic nucleotide pools that have different functions (Jurevicius and Fischmeister 1996, Stangherlin et al. 2011, Zaccolo et al. 2000). Cyclic AMP and GMP signaling occurs via the formation of cyclic nucleotide signalosomes. In these signalosomes the cyclic nucleotide effectors [protein kinase A and G (PKA and PKG), exchange factor directly activated by cAMP (EPAC) or cyclic nucleotide-gated ion channels] and PDEs form complexes through protein-protein interaction with each other and/or with scaffolding proteins, such as AKAPs and RACK1 (receptor of activated protein kinase C1) (Conti and Beavo 2007, Dodge-Kafka et al. 2005). As partners of signalosome complexes, PDEs modulate the diffusion and turnover of cAMP and cGMP gradients and regulate the diffusion of cyclic nucleotide signals into neighbor compartments (Conti and Beavo 2007).

PDEs integration into different signalosome complexes within various functional compartments has revealed the functional role of PDEs and linked them to the regulation of specific signaling pathways and biological responses. The expanding knowledge of PDE interactions has also opened possible therapeutic opportunities, but it also brings out additional challenges and questions in the development and design of novel therapeutics.

2.2 Regulation of PDEs

Cyclic nucleotide signaling through G protein–coupled receptors (GPCRs) is already well understood, and the mechanism that control the intensity, duration, and propagation of the signals emanating from the receptors have been uncovered. Phosphorylation of GPCRs by PKA, PKC and G protein-coupled receptor kinases (GRK) causes uncoupling of the receptor from the transducer G

proteins and reassembly of the macromolecular complexes that control receptor signaling and trafficking (Perry and Lefkowitz. 2002, Reiter and Lefkowitz. 2006). Interaction of G proteins with the regulators of G protein signaling (RGS) and the effector adenylyl cyclases also influences AMP homeostasis, and each step in the cascade is regulated by many negative feedback mechanisms (Hollinger and Hepler. 2002).

In addition to the regulation mechanisms that control signaling from GPCR, an increase in the cell cAMP level invariably activates PDEs. Several PDEs are phosphorylated by PKA, including the PDE3 and PDE4 families. PKA-mediated activation of PDEs leads to a rapid decrease of cAMP, and this has been shown to be one of the major factors affecting the cell cAMP levels (Omori and Kotera. 2007). A similar regulation operates with cGMP and PDE5. Control of the PDE5 activity occurs through PKG activation and cGMP binding. cGMP binding to the GAF domains of PDE5 stimulates phosphorylation by PKG and further increases both the affinity of the binding site in PDE5 for cGMP and the catalytic activity (Corbin et al. 2000, Kotera et al. 2003). Within a longer time frame, prolonged PKA activation causes phosphorylation of the cAMP response element-binding protein (CREB), which activates the transcription of PDE4 mRNAs (D'Sa et al. 2002).

The PDE1 family proteins are stimulated by Ca^{2+}/calmodulin (CaM) binding to the N-terminal site of PDE1s. PKA and CaM kinase II-dependent phosphorylation reduces the binding affinity of PDE1A and PDE1B (Florio et al. 1994, Hashimoto et al. 1989). Ca^{2+}/phospholipid-dependent protein kinase (PKC) activates PDE2A in the liver Golgi-endosomal fraction, and phosphorylation of rat PDE2A by the associated protein kinase inhibits PDE activity (Bentley. 2005, Geoffroy et al. 1999).

2.3 PDEs as a therapeutic target

Mutations in the genes that encode individual PDEs, and mutations in the genes encoding proteins involved in the function or expression of PDEs may both be associated with several human disease. For example, PDE6 mutations are associated with autosomal recessive retinitis pigmentosa (Hartong et al. 2006), whereas PDE4 mutations correlate with ischemic stroke and schizophrenia (Fatemi et al. 2008, Gretarsdottir et al. 2003). PDE8 and PDE11 mutations are associated with human adrenal adenomas and bilateral micronodular adrenal hyperplasia, respectively (Horvath et al. 2006, Horvath et al. 2008). Disorders in cyclic nucleotide signaling have also been shown to affect neoplasm formation, progression and metastases (Savai et al. 2010). Especially PDE4 and PDE5 families have

been evaluated as potential anticancer drug targets in experimental models. For example, inhibition of certain phosphodiesterases may have antitumor activity in acute myeloid leukemia and chronic lymphocytic leukemia (Lerner and Epstein. 2006, Ogawa et al. 2002). PDE4 inhibition suppressed cancer growth in a brain tumor model and in a prostate cancer xenograft mouse model (Goldhoff et al. 2008, Powers et al. 2015).

Many PDE-selective inhibitors have been developed, but only few have been approved for clinical use. This is mainly because of the off-target effects that limited their use. Although PDE-inhibitors predominantly inhibit specific PDE-families, and not all PDEs, they may still inhibit all family member proteins, often producing unwanted effects.

Most of the PDE-inhibitors have been developed against the PDE3, PDE4 and PDE5 families. PDE3 inhibitor milrinone is approved for the treatment of acute heart failure (Movsesian and Kukreja 2011). Cilostazol, another PDE3 inhibitor, is used to treat intermittent claudication (Dawson et al. 1998). PDE4 inhibitors are used especially in treatment of pulmonary diseases, such as asthma, chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis and allergic rhinitis (Rabe 2011, Tenor et al. 2011, Torphy. 1998). Sildenafil (Viagra®; Pfizer), a PDE5 inhibitor, is the most well-known agent, and likely the biggest success of all PDE inhibitors. During unsuccessful clinical trials studying the role of sildenafil as the treatment of angina and coronary artery disease, responses in off target tissues were observed, and the focus of sildenafil research changed to treatment of erectile dysfunction and later to the treatment of pulmonary hypertension (Ghofrani et al. 2006).

2.4 The PDE3 family

PDE3 family is consist of two genes, *PDE3A* and *PDE3B*, which are able to hydrolyze both cAMP and cGMP. cGMP behaves also as a competitive inhibitor for cAMP hydrolysis, which is why the PDE3 family is called the cGMP-inhibited PDE family (Degerman et al. 1997, Maurice and Haslam. 1990). The PDE3 family has two unique characteristics, a 44-aminoacid insert in the catalytic domain, and the N-terminal hydrophobic membrane association domains (Degerman et al. 1997).

PDE3A has two different transcripts, PDE3A1 and PDE3A2, which encode three N-terminal variant forms. PDE3A1 encodes PDE3A-136 (136 kDa) with two N-terminal hydrophobic membrane-associated regions (NHR1 and NHR2) and a catalytic domain. PDE3A-118 (118 kDa,) and PDE3A-

94 (94 kDa) are encoded by the PDE3A2 (Wechsler et al. 2002). PDE3A is expressed in the cardiac tissues, smooth muscle and platelets, and is involved in the regulation of the blood pressure, cardiac function and oocyte meiosis and platelet aggregation (Beavo. 1995, Begum et al. 2011). PDE3B has only one transcript. PDE3B is expressed in cells that are important in energy homeostasis, such as adipocytes and hepatocytes (Beavo 1995). PDE3B is of importance especially in insulin signalling (Nilsson et al. 2006).

The NHR regions impact greatly the intracellular localization of the PDE3 proteins. PDE3A-136, carrying two NHRs, is found only membrane bound, whereas PDE3A-118 containing NHR2 and PDE3A-94 carrying no NHR are found in both cytosolic and membrane-bound fractions (Wechsler et al. 2002). Both PDE3A and PDE3B with two NHRs are localized in the endoplasmic reticulum (Shakur et al. 2000).

PDE3s are phosphorylated at various sites on the N-terminal region by PKA, PKB and PKC (Degerman et al. 1997, Pozuelo Rubio et al. 2005). A few phosphorylation dependent interactions of PDE3A and PDE3B with the signaling regulator 14-3-3 have been described (Hunter et al. 2009, Onuma et al. 2002, Palmer et al. 2007, Pozuelo Rubio et al. 2005, Vandeput et al. 2013). In human embryonic kidney cells, PDE3A1 is phosphorylated primarily at S312 in response to PKA activation and PDE3A2 at S428 in response to PKC activation (Vandeput et al. 2013). Individual PDE3 isoforms may also integrate into different complexes in the same cell after phosphorylation. The mechanism of phosphorylation determines the incorporation PDE3s into different protein complexes (Ahmad et al. 2007, Ahmad et al. 2009, Vandeput et al. 2013).

2.5 Anagrelide

Anagrelide (6,7-dichloro-1,5-dihydroimidazo[2,1-*b*]quinazolin-2(3*H*)-one) (Figure 3), is a potent blood platelet reducing agent that is used for the treatment of essential thrombocytosis (Silverstein et al. 1988). Anagrelide was originally developed as an antiplatelet drug, and the thrombocytopenic effect was observed in preclinical studies (Abe Andes et al. 1984, Fleming and Buyniski 1979). The platelet-lowering effect of anagrelide results from the inhibition of megakaryocyte maturation and reduced proplatelet formation (Espasandin et al. 2015). Anagrelide represses GATA-1, FLI-1 and FOG-1, which are key megakaryocyte transcription factors and essential for the maturation and differentiation of megakaryocytes, suggesting that anagrelide exerts its effects upstream of GATA-1

and FOG-1 (Ahluwalia et al. 2010, Rinaldi et al. 2008). Gene expression studies have revealed that anagrelide regulates the expression of several genes. Anagrelide has been proposed to induce phosphorylation of eIF2a, an upstream regulator of the transcription factor ATF4, and thus increase the levels of ATF4 protein (Ahluwalia et al. 2015).

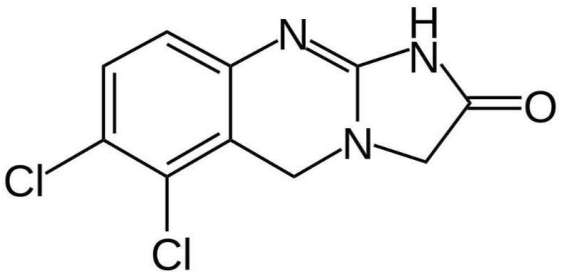

Figure 3. The structure of anagrelide.

Anagrelide also inhibits the function PDE3 (Gillespie. 1988). Stimulation of cAMP signaling blocks megakaryocyte maturation and differentiation (Freson et al. 2008). Megakaryocyte inhibition by cAMP is mediated by PKA-dependent down-regulation of the transcription factor E2A and its target CDKN1A (p21) (Rubinstein et al. 2012). Anagrelide represses this regulatory E2A/p21 loop in a similar manner, which suggests the involvement of cAMP in anagrelide's mechanism of action. However, other commercially available PDE3 inhibitors seem to have no effect on *in vitro* megakaryocyte maturation arguing against the participation of cAMP in anagrelide-induced platelet reduction (Wang et al. 2005). Inhibition of the platelet function is achieved at higher anagrelide concentrations that are required for megakaryocyte function inhibition (Espasandin et al. 2015).

Two different brands of anagrelide are available commercially. Xagrid® (Shire Pharmaceuticals) is used for the treatment of essential thrombocythemia. Thromboreductin® (AOP Orphan Pharmaceuticals AG) anagrelide hydrochloride is absorbed more slowly, which results in a lower maximum serum concentration (Cmax) in the plasma (Petrides et al. 2009). Anagrelide is metabolized into two major metabolites: 6,7-dichloro-3-hydroxy-1,5 dihydro-imidazo[2,1-b]quinazolin-2-one (BCH24426) that influences megakaryocytic differentiation as anagrelide, and 2-amino-5,6-dichloro-3,4-dihydroquinazoline (RL 603) (Wang et al. 2005).

3. Integrins

The integrin receptor family was discovered about 30 years ago, and is one of the best-understood cell adhesion receptors. Besides their role as major adhesion receptors, integrins have multiple roles also in the cell signaling and in the regulation of cell growth, survival, differentiation, division, migration, and apoptosis (Hynes. 2002). In tumor biology, the role of integrins in cell migration and invasion is probably the most studied function of integrins.

Integrins consist of alpha (α) and beta (β) subunits, and in mammals 18 α and 8 β subunits form 24 different integrins that bind to distinct subsets of the extracellular matrix (ECM) ligands (Figure 4) (Giancotti and Ruoslahti. 1999). Integrins mediate most of the effects of ECM through binding to various ECM components, organizing the cytoskeleton and activating intracellular signaling pathways (Hynes. 2002). Integrins transmit both chemical and mechanical signals across the plasma membrane in both directions (Zamir and Geiger. 2001).

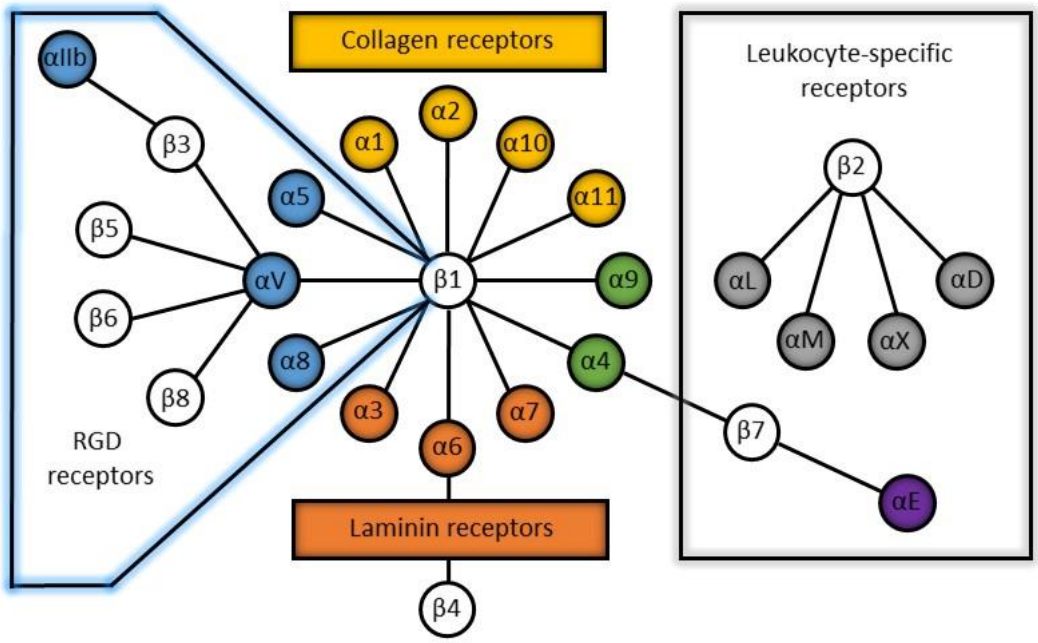

Figure 4. The integrin receptor families and their αβ associations. 18 α subunits can assort with 8 β subunits to form 24 distinct integrins. Adapted from Hynes, 2002.

3.1 Integrin functions

Clustering of integrin heterodimers into oligomers and changes in the conformation of individual heterodimers influence the binding of ligands (Carman and Springer 2003). Both of these are likely important for integrin function and their participation might vary depending on the integrin, cell type and biology circumstances. Integrin ectodomains can have bend "closed" conformation, intermediate extended conformation and extended "open" conformation (Nishida et al. 2006). These correspond to low affinity, activated, and activated and ligand occupied conformations on cells, respectively (Mould and Humphries 2004, Takagi et al. 2002). In integrin clustering the heterodimers interact with each other to form hetero-oligomers. This can be caused by inside-out signals that stimulate the recruitment of multivalent protein complexes to integrin cytoplasmic domains (Critchley and Gingras 2008, Wu 2005) or by the release of cytoskeletal constraints leading to the free diffusion of integrins in the plane of the membrane (Kucik 2002).

Integrin α- and β-subunits are typical type I transmembrane proteins that have the amino terminus outside the cell, a single transmembrane domain and a carboxy-terminal cytoplasmic tail (Lau et al. 2008). Integrin cytoplasmic domain interactions with each other or cytoplasmic proteins lead to allosteric rearrangements leading to integrin activation and inside-out signaling (O'Toole et al. 1994). This inside-out signaling is activated especially by talin, an adaptor protein that induces a conformational switch in the integrin extracellular domain that leads to receptor activation and increased affinity for ECM ligands (Tadokoro et al. 2003). In addition to talins, kindlins also co-activate integrins and talin-1 (Ma et al. 2008). Extracellular factors, including ECM ligands and ligand binding to integrins can directly activate integrins triggering the outside-in signaling (Hynes. 2002). Inside the cell enzymes such as FAK/c-Src complex, Ras and Rho GTPases and adapters such as Cas/Crk and paxillin are key mediators of integrin downstream signaling. These assemble within dynamic adhesion structures, including focal adhesions, focal complexes and podosomes (Linder and Kopp. 2005, Zaidel-Bar et al. 2004).

3.2 Integrins in cancer

The role of integrins in cell migration and invasion is well established. However, integrins can also regulate cell proliferation (Assoian and Klein. 2008). Recent studies have illustrated the crucial, but at same time inconsistent, role of integrins in the regulation of tumor cell survival. In addition to their

ligation dependent effects, in some cases unligated integrins can also control the tumor cell survival positively or negatively, thereby affecting tumor metastases and growth. Besides their role in tumor cells, integrins on the surface of tumor-associated cells such as endothelial cells, fibroblasts and inflammatory cells can considerably influence the malignant behavior of the tumor (Desgrosellier and Cheresh. 2010).

Various integrins contribute to tumor progression. Increased expression of integrins such as αvβ3, αvβ5, α5β1, α6β4, α4β1 and αvβ6 correlates with disease progression in several tumor types (Desgrosellier and Cheresh. 2010). Integrins are able to either enhance cell survival or initiate apoptosis, depending on the environmental state. Several mechanisms, including increased expression of apoptosis regulator proteins Bcl-2 and FLIP, activation of the PI3K-AKT pathway, nuclear factor-κB (NF-κB) signaling and p53 activation, may enhance cell survival after integrin ligation (Aoudjit and Vuori. 2001, Aoudjit and Vuori. 2001, Bao and Stromblad. 2004, Matter and Ruoslahti. 2001). Depending on specific integrin-growth factor receptor pairs, these pathways are variably regulated.

The role of integrins in regulating apoptosis is variable, suggesting a possible dual role for integrins in both promoting cell survival and inducing cell death. Some integrins such as αvβ3 and α6β4, enhance tumor progression while for example α5β1 inhibits oncogene-induced transformation (Giancotti and Ruoslahti. 1990, Petitclerc et al. 1999, Varner et al. 1995). In some mouse models of glioblastoma and melanoma αvβ3 integrin could inhibit tumor progression (Danen et al. 1996, Kanamori et al. 2004). These differences, can in some respect, be explained by the ligation status of integrins (Stupack et al. 2001, Zhao et al. 2005). Integrins do not function as oncogenes, because they lack the ability to transform cells. However, some integrins co-operate with receptor tyrosine kinases or oncogenes to enhance tumorigenesis. Integrin α6β4 co-operation with ERBB2 increases breast tumor formation and invasion, whereas integrin α1 is required for KRAS-G12D-induced lung tumor genesis (Guo et al. 2006, Macias-Perez et al. 2008). Integrin αvβ3 might increase the tumorigenic potential of melanomas and various carcinomas (Huveneers et al. 2007). Integrins also seem to co-operate with various growth factor and cytokine receptors, including epidermal growth factor (EGF) receptors, hepatocyte growth factor (HGF) receptors, vascular endothelial growth factor (VEGF) receptors and fibroblast growth factor (FGF) receptors (Bertotti et al. 2005, Byzova et al. 2000, Guo et al. 2006, Miyamoto et al. 1996, Nikolopoulos et al. 2004).

3.3 Integrin alpha 4 (ITGA4)

The integrin alpha 4-chain (CD49d) associates with either the β1 chain (CD29) or the β7 chain forming α4β1 (very late antigen-4, VLA-4) and α4β7 (lymphocyte Peyer patch adhesion molecule, LPAM) integrins, respectively (Hynes. 2002). The alpha 4 integrins mediate especially cell-cell adhesions that are crucial to the immune function (Kinashi. 2012). They are involved in myogenesis, hematopoiesis, and cardiac and placental development (Arroyo et al. 1996, Rosen et al. 1992, Yang et al. 1995). The alpha 4 integrins are also involved in the pathogenesis of some cardiovascular diseases (Liu et al. 2000). α4β1 binds to the vascular cell adhesion molecule-1 (VCAM-1) that is expressed on the surface of endothelial and stromal cells and to fibronectin in the extracellular matrix, whereas α4β7 binds to the mucosal vascular addressin cell adhesion molecule-1 (MAd-CAM-1) (Goodman and Picard. 2012).

ITGA4 expression is dependent on the development status in the myocytes, placental and fibroblastic cells (Liu et al. 2000). α4 integrins have a central role in leukocyte trafficking, and they are expressed during hematopoiesis on early pluripotent progenitor cells, mononuclear leukocytes, and eosinophils (Arroyo et al. 1999). These integrins are involved in the pathogenesis of several chronic diseases such as multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases, and contact hypersensitivity (Abraham et al. 1994, Chisholm et al. 1993, Yednock et al. 1992).

Humanized monoclonal antibody natalizumab, that targets ITGA4 has been approved for the treatment of multiple sclerosis and Crohn's disease by the U.S. Food and Drug Administration (FDA) (Polman et al. 2006, Rutgeerts et al. 2009). In a xenograft model of primary leukemia, natalizumab prolonged survival of mice when treated with the combination of vincristine, dexamethasone, and l-asparaginase plus natalizumab (Hsieh et al. 2013). In addition, natalizumab decreases myeloma growth by blocking the interaction between myeloma cells and bone marrow stromal cells (Podar et al. 2011).

The expression of α4β1 integrin has been associated with poor prognosis in a few cancer types including chronic lymphocytic leukemia and neuroblastoma (Buggins et al. 2011, Bulian et al. 2014, Young et al. 2015). Increased expression of α4β1 integrin has also been linked with ovarian cancer progression (Slack-Davis et al. 2009). Inhibition of ITGA4 in MV3 melanoma cells with heparin derivatives decreases melanoma metastatic potential and interaction between VCAM-1 on activated

endothelial and ITGA4 in melanoma cells enhances the metastatic capacity of melanoma cells (Klemke et al. 2007, Schlesinger et al. 2012).

α4 integrins can activate critical pathways downstream of integrins, including FAK, Src, Akt and ERK (Wu et al. 2008). The focal adhesion kinase (FAK) is one of the key mediators of integrin signalling and a promoter of cell migration, and is highly expressed in malignant GISTs (Koon et al. 2004, Mitra and Schlaepfer. 2006). Interestingly, imatinib stimulated the SRC family kinase (SFK) and FAK activation and integrin signaling in a mouse $Kit^{V558\Delta/+}$ GIST model (Rossi et al. 2010).

3.4 Integrins as targets for cancer therapy

The central role of integrins in cell attachment, migration, invasion, survival and angiogenesis has attracted attention also as a potential anticancer target. Anti-integrins are usually used in combination with other anticancer drugs and may have potential as imaging agents in cancer diagnosis (Cox et al. 2010). Several synthetic peptides and humanized antibodies have been developed to interfere integrin functions. Even though the *in vitro* and preclinical results have been encouraging, the late phase clinical trials have, however, been mostly disappointing (Desgrosellier and Cheresh. 2010). For example, in a recent POSEIDON trial no improvement in efficacy was seen when treating patients with K-RAS wild-type metastatic colorectal cancer with an anti-av-integrin antibody (abituzumab) combined with the standard of care (Elez et al. 2015). Similarly, in a phase 3 trial the av-integrin antagonist (cilengitide) in combination with radiotherapy did not improve glioblastoma patients' survival (Stupp et al. 2014). Integrin function complexity and integrin-growth factor receptor cross-talk makes integrin targeting challenging, and some signaling molecules downstream of integrins might be more beneficial therapeutic targets. However, growing knowledge of the complex biological roles of integrins in cancer promotes the design of better integrin targeting agents and may improve the stratification of patients that are most likely to respond therapies.

4. The snail family of zinc-finger transcription factors

The snail superfamily is involved in enhancing of cell migration during both the embryotic development and tumor progression. The snail family members have also found to be important in the signaling cascades that affect the left-right identity, neural differentiation, cell division and cell survival.

The snail family members are essential for mesoderm formation in many organisms, and they are also involved in processes that require larger scale cell movements such as neural crest formation (Alberga et al. 1991, LaBonne and Bronner-Fraser. 2000, Nieto et al. 1994). The role of these transcription factors is of importance especially in promoting cell process that is generally known as the epithelial-mesenchymal transition (EMT) (Batlle et al. 2000, Cano et al. 2000). In this mechanism, epithelial cells loose cell adhesion and polarity, may obtain migratory properties, dissociate from the epithelium and become mesenchymal cells.

4.1 Structure and regulation

Snail was described first in *Drosophila melanogaster*, where it was shown to be essential for the mesoderm formation (Grau et al. 1984, Nusslein-Volhard et al. 1984). Snail was also found in vertebrates, including humans, and non-vertebrates like insects and nematodes (Peinado et al. 2007). In vertebrates three snail family protein have been identified: Snail1 (Snail), Snail2 (SLUG) and Snail3 (SMUC). The snail family members encode zinc-finger type transcription factors. They all share a similar structure with a highly conserved C-terminal region containing four to six zinc-fingers, and a more complicated N-terminal region. Zinc fingers bind to the E-box motif (5′-CANNTG-3′), indicating that they might compete with the basic helix-loop-helix (bHLH) transcription factors for the binding on the same target gene promoters (Cano et al. 2000, Kataoka et al. 2000).

The snail family members are proposed to act as transcriptional repressors on E-box binding (Batlle et al. 2000). The repressor activity depends not only on the finger region but also on the so called SNAG (Snail/Gli) domain (Grimes et al. 1996). The SNAG domain is conserved in vertebrate *Snail* genes, but, for example, in *Drosophila* Snail does not contain the SNAG. The repressor activity in Drosophila is mediated through an interaction with C-terminal binding protein (P-DLS-K) (Nibu et al. 1998). A nuclear export sequence (NES) and a serine rich domain (SRD) in the central region of

Snail are important in the subcellular localization and regulation of Snail stability, respectively (Dominguez et al. 2003).

Snail interacts with several co-repressor complexes, such as Sin3A and the histone deacetylase 1/2 (HDAC1/2) complex, the protein arginine methyltransferase 5 (PRMT5)/Ajuba complex, the polycomb repressive complex 2 (PRC2), the 14-3-3 complex, the lysine-specific demethylase 1 (LSD1)/CoREST and the suppressor of variegation 3-9 homolog 1 (Suv39H1) complex (Dong et al. 2013, Herranz et al. 2008, Hou et al. 2008, Hou et al. 2010, Lin et al. 2010, Peinado et al. 2004). The Snail activity is mainly regulated through the central part of the protein that contains most of the sites for post-transcriptional modification, including a serine rich domain (SRD), the nuclear export sequence (NES) and two lysine oxidation sites. Glycogen synthase kinase-3β (GSK-3β) is responsible for the phosphorylation at the SRD region, whereas the small C-terminal domain phosphatase (SCP) removes these phosphorylations (Wu et al. 2009, Zhou et al. 2004). The F-box/WD repeat-containing protein 1A (β-TrCP1) and the F-box/LRR-repeat protein 14 (FBXL14) are responsible for the degradation, ubiquitination and protease degradation of Snail (Vinas-Castells et al. 2010, Zhou et al. 2004). The sequence-specific interactions with DNA are mediated via the C-terminal regions that are also responsible for the repressor activity of Snail. The nuclear accumulation and the repressor activity of Snail in the nucleus are promoted by Serine/Threonine-Protein Kinase PAK 1 phosphorylation (Yang et al. 2005).

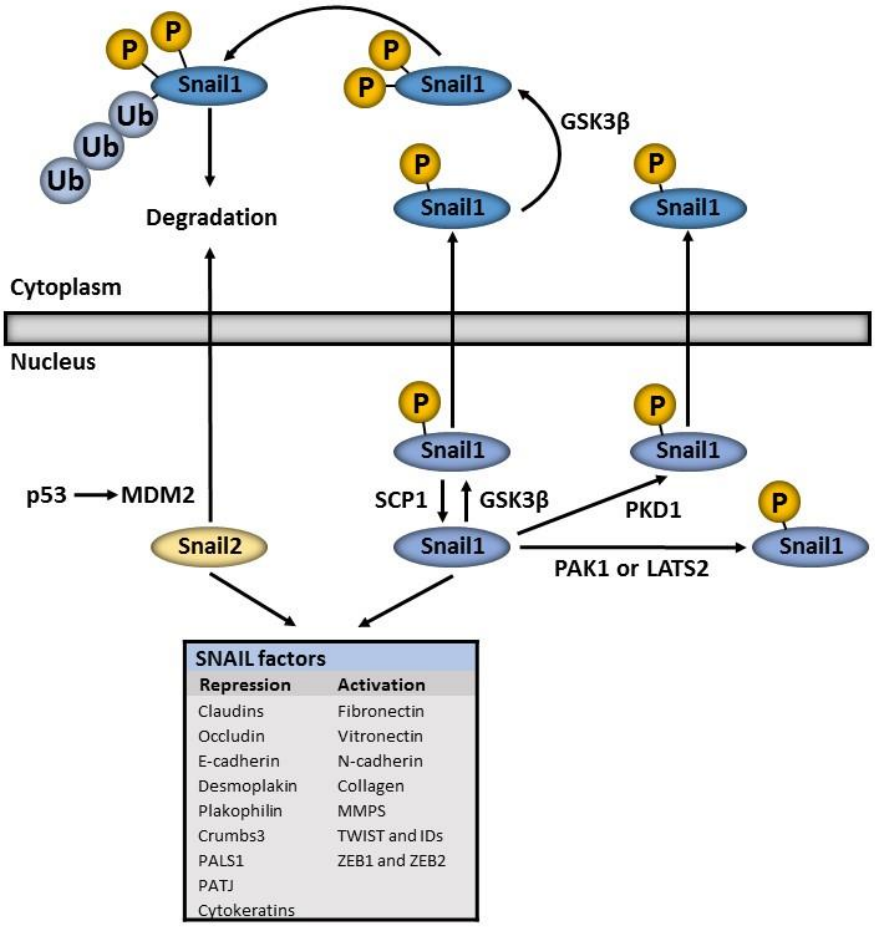

Figure 5. Regulation of Snail transcription factors in epithelial-mesenchymal transition. Glycogen synthase kinase-3β (GSK3β) phosphorylates (P) Snail1 that facilitates the nuclear export of Snail1, and enables the ubiquitin (Ub)-mediated degradation of Snail1. Protein kinase D1 (PKD1) phosphorylates Snail1 that leads also to its nuclear export. Conversely, phosphorylation of Snail1 by p21 activated kinase 1 (PAK1) or large tumor suppressor 2 (LATS2), or dephosphorylation of Snail1 by small C-terminal domain phosphatase 1 (SCP1) enhances the activity of Snail1 and promotes its nuclear retention. Snail2 is degraded by p53-mediated recruitment to the p53–mouse double minute 2 (MDM2) complex. Adapted from Lamouille *et al*. 2014.

Many signaling molecules, such as the transforming growth factor-β (TGF-β), the hepatocyte growth factor (HGF), the fibroblast growth factor (FGF), the epidermal growth factor (EGF), the tumor necrosis factor-α (TNF-α), and several cytokines may induce Snail expression in various cellular contexts (Nieto. 2002). FGF and EGF, for example, activate receptor tyrosine kinase (RTK) signaling

that eventually results in the induction of Snail (Ciruna and Rossant. 2001, Lu et al. 2003), whereas the Nf-κB pathway regulates Snail expression through transcriptional and post-transcriptional mechanisms. Nf-κB binds to the Snail promoter region and increases the transcription of Snail (Barbera et al. 2004). Interestingly, Snail can also bind and repress its own promoter, indicating the existence of an autoregulatory loop (Peiro et al. 2006).

4.2 Epithelial mesenchymal transition (EMT)

In epithelial–mesenchymal transition (EMT) epithelial cells transdifferentiate into motile mesenchymal cells. It is essential in the fetal development, stem cell behavior, wound healing, and EMT contributes to cancer progression and fibrosis. Snail, the zinc-finger E-box-binding (ZEB) transcription factor and the basic helix–loop–helix (bHLH) transcription factor are the key switches in cell differentiation and EMT.

In EMT, epithelial cells lose their junctions and apical-basal polarity, reorganize their cytoskeleton and change the signaling programs defining the cell shape and gene expression that further increases the motility of cells and enables the development of an invasive phenotype (Thiery et al. 2009). Variations in EMT-associated gene expression are dependent on tissue and cell type and on the degree of progression towards mesenchymal differentiation. One of the hallmarks of EMT is the downregulation of E-cadherin (encoded by *CDH1*) to reinforce the destabilization of adherens junctions (Peinado et al. 2007). E-cadherin is considered a suppressor of invasion during cancer progression (Birchmeier and Behrens. 1994). In addition, the repression of genes encoding desmoplakin and plakophilin, and claudins and occludin stabilizes the dissolution of desmosomes and apical tight junctions (Huang et al. 2012). These changes prevent the reformation of epithelial cell-cell junctions and result in the loss of the epithelial barrier function (Peinado et al. 2007).

The Snail transcription factor is one of the master regulators of the gene expression that contributes to the repression of the epithelial phenotype and activates the mesenchymal phenotype. Of the three Snail proteins found in the vertebrates, Snail1 (Snail) and Snail2 (SLUG) activate the EMT during the fetal development, and in cancer and fibrosis (Barrallo-Gimeno and Nieto. 2005). Snail or SLUG, by binding to the E-box DNA sequences through their carboxy-terminal zinc-finger domain, suppress the epithelial genes (Cano et al. 2000). In brief, upon binding to the proximal promoter region of E-cadherin, Snail1 recruits the Polycomb repressive complex 2 (PRC2), methyltransferase G9a and

suppressor of variegation 3–9 homologue 1 (SUV39H1), and all of these components coordinate histone modifications, specifically methylation and acetylation (Dong et al. 2012, Dong et al. 2013, Herranz et al. 2008). In addition to suppression of epithelial genes, Snail activates genes such as ZEB factors, N-cadherin and matrix metalloproteinases (MMP) that contribute to the mesenchymal phenotype (Peinado et al. 2007).

Various signaling pathways acting through RTKs cooperate in the progression of EMT, often activating Snail1 expression. Snail1 and Snail2 also cooperate with other transcription regulators to control gene expression (Jorda et al. 2005, Vincent et al. 2009). Post-translational modifications also control the localization, degradation and activation of Snail (Peinado et al. 2007). GSK3β-mediated Snail1 phosphorylation inactivates its transcriptional activity, and several pathways including WNT and PI3K-AKT pathways increase Snail1 activity by influencing GSK3β-mediated phosphorylation (Yook et al. 2006, Zhou et al. 2004). In addition, Notch and NF-κB signaling interfere the GSK3β–Snail1 interactions (Sahlgren et al. 2008, Wu et al. 2009).

4.3 Snail2 (SLUG)

SLUG was first identified in the neural crest and in the developing mesoderm in the chick embryos (Nieto et al. 1994). This translational repressor is best known to control EMT and promote cancer invasion and metastasis. However, SLUG has also a role in cell cycle progression (Emadi Baygi et al. 2010, Liu et al. 2010, Turner et al. 2006, Wang et al. 2015). SLUG is expressed in several types of human cancer, and its expression is often associated with various prognostic factors of unfavorable outcome (Cobaleda et al. 2007, Wang et al. 2013).

SLUG is expressed in migratory neural crest cells, but not in premigratory neural crest cells (Jiang et al. 1998). It is required for melanoblast migration and survival (Perez-Losada et al. 2002). It is also expressed in the developing bone, craniofacial mesenchyme, endocardial cushions and cardiac outflow tracts, and in the mesenchymal components of the lungs, kidneys and gut (Oram et al. 2003). SLUG-deficient mice are viable, though they have a white forehead blaze and other pigmentation abnormalities in the ventral body, the tail and feet, and macrocytic anemia and infertility, indicating an essential role for SLUG in the hematopoietic stem cells, germ cells and melanocytes (Perez-Losada et al. 2002). Similarly, deletions of *SLUG* in the human cause the Waardenburg disease, a rare disorder associated with abnormal pigmentation and sensorineural deafness (Sanchez-Martin et al.

2003). Interestingly, dysfunction of KIT may cause piepaldism and sensorineural deafness (Spritz and Beighton. 1998). Targeted *SLUG* deletions in mice have also phenotypic similarities with mice with defective KIT or stem cell factor (SCF), including abnormal pigmentation, and gonadal and hematopoietic defects (Motro et al. 1991, Perez-Losada et al. 2002).

The evidence for the occurrence of mesenchymal-to-epithelial transition (MET) in sarcomas has been obtained in several studies. SYT-SSX1 and SYT-SSX2 fusion proteins, typical for synovial sarcoma, are able to interact with Snail or SLUG, preventing their suppressive effects on E-cadherin expression and leading to the acquisition of epithelial features indicative of mesenchymal-to-epithelial transition (Saito et al. 2006). SLUG has been identified as a negative regulator of E-cadherin expression in integrated proteomics and genomics analyses in soft tissue leiomyosarcomas. In these analyses, the knockdown of SLUG increased E-cadherin and decreased vimentin expression, which was associated with decreased cancer cell proliferation and invasion (Yang et al. 2010).

In some types of human cancer, such as lung cancer, esophageal cancer and colorectal cancer, patients with SLUG-positive cancer generally have poor outcome (Shioiri et al. 2006, Uchikado et al. 2005, Wu et al. 2015). SLUG downregulation decreases cell viability and inhibits invasiveness in preclinical models of prostate cancer and neuroblastoma (Emadi Baygi et al. 2010, Vitali et al. 2008). SLUG silencing sensitizes ovarian cancer cells to cisplatin, and SLUG might have a role in the signaling network that drives survival and imatinib resistance of Bcr-Abl-expressing cells (Haslehurst et al. 2012, Mancini et al. 2010).

AIMS OF THE STUDY

The aims of the thesis were:

1. To investigate the clinical significance of SLUG, ITGA4, PDE3A and PDE3B expression and their association with patient and tumor characteristics and survival in GIST.

2. To study the effects of SLUG, ITGA4 and PDE3 knockdown, and ITGA4-specific and PDE3-specific inhibitors on GIST cell proliferation and invasion.

3. To investigate the efficacy of a PDE3 inhibitor, anagrelide, in patient-derived GIST xenograft mouse models.

MATERIALS AND METHODS

1. Patient and tumor samples (I, II, III)

The associations of GIST with potential biomarker expression, tumor features and patient survival outcomes were investigated in three clinical cancer patient series. The first series consisted of tissue samples from 630 tumors of 36 histological tumor types including samples from 55 GISTs. This tumor series was retrieved and identified from the archives of the Department of Pathology, Helsinki University Hospital.

The second series is a population-based cohort consisting of GIST patients who were treated with surgery in the Western Sweden from 1983 through 2000 (Nilsson et al. 2005). Out of the total of 288 patients in the cohort, we included in the study on SLUG 187 (64.9%) tumor tissue samples, and in the study on ITGA4 147 (51.0%) tumor samples. The included patients had tumor tissue available, tumor histology was compatible with GIST and stained positively for KIT in immunohistochemistry, and we also provided that the patients had information about gender, tumor diameter, and follow-up available. None of the patients was treated with imatinib or other tyrosine kinase inhibitors after surgery for GIST.

The third series consisted of GIST patients who were entered to the Scandinavian Sarcoma Group (SSG) XVIII/Arbeitsgemeinschaft Internistische Onkologie (AIO) adjuvant trial (Joensuu et al. 2012, Joensuu et al. 2016). SSGXVIII/AIO is an open-label, multicenter, phase 3 study. The patients had KIT-positive, operable GIST and were at a high risk for GIST recurrence estimated according to the modified National Institute of Health (NIH) Criteria (Joensuu. 2008). After surgery, the patients were randomly allocated to receive adjuvant imatinib 400 mg per day orally either for 12 or 36 months. A total of 400 patients from Finland, Germany, Norway and Sweden were entered to the trial between February 2004 and September 2008. Representative tumor tissue for the SLUG study was available from 313 (85.1%) out of the 368 patients. Each patients had localized disease at the time of randomization. The median follow-up time after the date of randomization was 6.1 years (Joensuu et al. 2016).

Institutional review committees of the Helsinki University Hospital, Helsinki, Finland, and Sahlgrenska University Hospital, Gothenburg, Sweden, approved the current studies on GIST biology. The SSGXVII/AIO trial was approved by the ethics boards of the participating hospitals and the national supervising authority in each country as per the national legislation.

2. Gene expression dataset (II, III)

Cancer and normal tissue mRNA expression data were derived from the MediSapiens *in silico* database of human transcriptomes that contains data from almost 20,000 human genes across 9,783 human tissue samples (ist.medisapiens.com). The gene tissue index (GTI) outlier statistics (Mpindi et al. 2011) was used to measure and rank the outlier genes that are highly expressed in GIST in comparison with histologically normal gastrointestinal tract tissue samples. The GTI is a modified version of the poverty index formula used in economics. A large positive GTI for a gene indicates an outlier in the disease group and a large negative GTI an outlier in the control group. The outlier analysis of mRNA expressions in the database was based on 6 GISTs and 34 histologically normal gastrointestinal tract tissue samples (study II) or 77 GISTs and 10,654 to 19,986 reference samples (study III). The results were filtered using identified drug target genes from https://www.drugbank.ca (Wishart et al. 2006).

3. High-throughput drug screening (III)

A total of 217 approved or investigational anti-cancer compounds were used in the study. The compounds were dissolved in DMSO and plated in 5 different concentrations covering a 10,000-fold concentration range into the wells of 384-well plates. GIST882 (1000 cells/well) and GIST48 cells (2000 cells/well) were plated on the compounds on the 384-well plates. The plates were incubated in a humidified environment at 37°C and 5% CO_2. Cell viability was measured after 72 h incubation using the CellTiter-Glo Cell Viability Assay (Promega Inc., Madison, WI, USA) and a PHERAstar FS plate reader (BMG Labtech, Ortenberg, Germany). The data were normalized to negative (DMSO) and positive (100 μmol/L benzethonium chloride) control wells. The Marquardt-Levenberg algorithm was used to estimate the 4-parameter logistic dose–response curves using the Breeze analysis platform (Yadav et al. 2014). Drug responses to the test compounds were measured using the drug sensitivity score (DSS). A high DDS indicates high responsiveness of the cells to a drug.

4. Immunohistochemistry (I, II, III)

Tissue microarrays were constructed from the representative parts of the formalin-fixed paraffin-embedded tumor tissues using a 0.6 mm (the mixed tumor series and the Western Sweden series) or a 1.0 mm diameter needle (the SSGXVIII/AIO series). Protein expressions were evaluated from 5 µm tissue microarray sections by immunohistochemistry. The sections were deparaffinised in xylene and hydrated in a graded series of alcohol. Prior to staining, hydrogen peroxide was used to block endogenous peroxidase activity. Antigen retrieval methods and antibodies used are represented in the Table 1. All antibody dilutions were done in a PowerVision preantibody blocking solution and incubated either 30 minutes at room temperature or overnight at 4°C (Table 1). Primary antibody binding was detected by using a BrightVision+ Histostaining kit (Immunologic BV, Duiven, The Netherlands) following the manufacturer's instructions. The slides were counterstained with Mayer´s hematoxylin. Expression of the studied proteins in tumor cells was scored as either negative (less than 10 % tumor cells stained, study III; less than 20 % tumor cells stained, study I) or positive; or categorized as a negative low or high expression based on staining intensity (study II). The immunostainings were analyzed blinded without knowledge of the clinical or histopathological data.

Table 1. List of antibodies used in immunohistochemistry.

Protein	Antibody	Dilution	Incubation time	Pre-treatment buffer	Manufacturer
DOG1	NCL-L-DOG-1	1:250	Overnight at +4°C	EDTA + water bath	Novocastra
ITGA4	A9385	1:100	30 min at RT	Sodium citrate + autoclave	LifeSpan BioSciences
KIT	A4502	1:300	Overnight at +4°C	Sodium citrate + water bath	DAKO
PDE3A	HPA014492	1:100	30 min at RT	Sodium citrate + autoclave	SIGMA
PDE3B	HPA024342	1:20	30 min at RT	Sodium citrate + autoclave	SIGMA
SLFN12	ab113238	1:50	Overnight at +4°C	Sodium citrate + autoclave	Abcam
SLUG	9585	1:50	Overnight at +4°C	Sodium citrate + autoclave	Cell Signaling Techn.

5. Quantitative PCR (II, III)

RNA was extracted from the FFPE tissue sections using a High Pure RNA Paraffin kit (Roche Diagnostics GmbH, Mannheim, Germany) and from the cell lines using the NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany). The mRNA was reverse transcripted to cDNA with a SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

PDE3A and *PDE3B* mRNA expression levels were quantified in 7 GIST tissue samples and in 30 leiomyosarcoma samples, two GIST cell lines (GIST882, GIST48) and GIST xenograft tumor samples. *ITGA4* mRNA expression levels were quantified in 13 GISTs, 29 leiomyosarcomas, 8 synovial sarcomas and 9 undifferentiated pleomorphic sarcomas. These qPCR samples were selected at random from the mixed tumor patient series.

PDE3A, PDE3B and ITGA4 mRNA expressions were quantitated with real-time PCR using hydrolysis probes (i.e., hybridization probes labelled with a reporter dye and a quenching dye) and a LightCycler 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany). cDNA was amplified in a 20 μL PCR mixture using LightCycler 480 Probes Master reagents (Roche Diagnostics GmbH, Mannheim, Germany) and fluorescein-labelled locked nucleic acid (LNA) hydrolysis probes (Probe 79 for PDE3A, Probe 10 for PDE3B and Probe 13 for ITGA4) or the LightCycler® Yellow 555-labeled LNA hydrolysis probes (Probe TBP for PDE3A and Probe G6PD for PDE3B and ITGA4) from a Universal ProbeLibrary Set (Roche Diagnostics GmbH, Mannheim, Germany). The PCR mixture contained 1× PCR buffer, 100 nmol/L of probe and 200 nmol/L of each primer. The primers (Table 2) and the probes were designed using the ProbeFinder program at Assay Design Center of Universal ProbeLibrary (www.universalprobelibrary.com; Roche Diagnostics GmbH).

The cycling parameters for PDE3A, PDE3B and ITGA4 were an initial denaturation step at 95°C for 10 minutes, followed by 45 cycles (PDE3A, PDE3B) or 60 cycles (ITGA4) with denaturation at 95°C for 15 seconds, annealing at 60°C for 45 seconds and elongation at 72°C for 45 seconds. The results were analysed using the Basic Relative Quantification method (Roche Diagnostics GmbH).

Table 2. The primers used in the quantitative PCR analyses.

ITGA4:	Forward: 5′-AGGAAGTTCCAGGTTACATTGTTT-3′
	Reverse: 5′-TTAGAAGAGAAATAGAATCTTGGTGGA-3′
PDE3A:	Forward: 5′- AAAGACAAGCTTGCTATTCCAAA -3′
	Reverse: 5′- GTGGAAGAAACTCGTCTCAACA -3′
PDE3B:	Forward: 5′- AACAATGGTATAAGCCTCATTATCAA -3′
	Reverse: 5′- CGAGCCTCATTTAGCACTGA -3′
TBP:	Forward: 5′ - TGAATCTTGGTTGTAAACTTGACC -3′
	Reverse: 5′ - CTCATGATTACCGCAGCAAA -3′
G6PD:	Forward: 5′- GAGCCAGATGCACTTCGTG -3′
	Reverse: 5′- GGGCTTCTCCAGCTCAATC -3′

6. Cell lines (I, II, III)

GIST882 and GIST48 cell lines were a kind gift from Doctor Jonathan A. Fletcher (Harvard Medical School, Boston, Massachusetts, USA). GIST-T1 cell line was purchased from Cosmo Bio (Tokyo, Japan). GIST882 (a primary human GIST cell line) harbours a homozygous missense mutation in *KIT* exon 13 encoding a p.K642E mutant oncoprotein. GIST48 (a GIST cell line that progressed after an initial response to imatinib) harbors a *KIT* exon 11 p.V560D missense mutation leading to p.V560D and a secondary exon 17 (kinase activation loop) missense mutation leading to p.D820A. GIST-T1 (established from a metastatic plural tumor from a GIST of the stomach) is a *KIT* exon 11 mutant cell line that has a heterozygous in-frame deletion of 57 bases. All cell lines were mycoplasma-tested and the authenticity of the cell lines was confirmed with DNA sequencing. The cells were cultured in a humidified 5% CO_2 atmosphere at 37°C. GIST882 and GIST48 cells were cultured in the RPMI 1640 medium (GIBCO, CA, USA), supplemented with 20% fetal bovine serum with 2% penicillin/streptomycin (GIBCO), and GIST-T1 cells were cultured in a DMEM medium (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum with 2% penicillin/streptomycin (GIBCO).

7. siRNAs (I, II, III)

GIST cell lines were transfected using the Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA). The cells were serum starved for six hours before transfection in an Opti-MEM Reduced Serum Medium (GIBCO). Transfections were done according to the manufacturer's instructions adding 5 pmol/L of siRNA onto the GIST882 and GIST48 cells, and 10 pmol/L of siRNA onto the GIST-T1 cells. Transfections were done with an ON-TARGET plus Human KIT siRNA, an ON-TARGET plus Human PDE3A siRNA, an ON-TARGET plus Human PDE3B siRNA, an ON-TARGET plus Human ITGA4 siRNA, and an ON-TARGET plus Human SNAI2 (SLUG) siRNA, which are pools of target-specific siRNAs (Thermo Scientific Dharmacon, Rockford, IL, USA). The ON-Target plus Non-Targeting Pool (Thermo Scientific Dharmacon) was used as a negative control

8. Inhibitors (II, III)

PDE3 inhibitors cilostazol, milrinone and amrinone were purchased from Sigma (St. Louis, MO, USA) and anagrelide hydrochloride was purchased from Lancrix (Shanghai, China). BIO1211 and

BIO5192, both integrin α4β1 (VLA-4)-specific inhibitors, were purchased from Tocris Bioscience (Bristol, UK). Imatinib was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Imatinib was reconstituted in water and BIO1211 in PBS. All other inhibitors were reconstituted in DMSO. Cell lines were cultured for 1 day, and then incubated with inhibitors for 1 to 144 hours. Final inhibitor concentration of the drugs varied from 0.001 μM to 100 μM.

9. cAMP and cGMP assays (III)

PDE3 activity changes in the GIST882 and GIST48 cell lines were analyzed indirectly by measuring the amount of cAMP and cGMP. GIST cells were treated with cilostazol, milrinone, amrinone, and anagrelide hydrochloride for 6 hours, lysed in 0.1 M HCl and the concentration of cAMP and cGMP was determined using a cAMP Enzyme Immunoassay kit or a cGMP Enzyme Immunoassay kit (SIGMA, St. Louis, MO, USA).

10. TUNEL assay (I, III)

GIST cell apoptosis was analyzed with a Click-iT TUNEL Alexa Fluor 488 Imaging Assay (Invitrogen, Carlsbad, CA, USA) after exposure to KIT siRNA, PDE3A siRNA, PDE3B siRNA, and SLUG siRNA and anagrelide hydrochloride, imatinib or anagrelide + imatinib combination. GIST882 and GIST48 cells were plated at a density of 10,000 cells/well or 15,000 cells/well, respectively, in 96-well plates. After 24 hours of culture the cells were treated with siRNAs or inhibitors. The TUNEL assay was performed 72 hours after siRNA transfections, or 24 hours after adding the drugs. Cells were photographed (magnification ×200) using an immunofluorescence microscope (Leica CTR6000, Leica microsystems, Bannockburn, IL, USA). The frequency of apoptotic cells was expressed as the average number of apoptotic cells per one microscope field.

11. Proliferation assay (I, II, III)

Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics, Indianapolis, IN, USA). For the assay, 20,000 GIST48 cells, 15,000 GIST882 cells, and 5,000 GIST-T1 cells were plated in 96-well plates. Cell proliferation was analyzed after adding 10 μL of the MTT reagent into each well and incubation for 4 hours at 37°C, after which 100 μL of the solubilization solution was added, and the cells were incubated at

37°C overnight. The plates were read with a Multiscan EX Microplate photometer (Thermo Scientific, Rockford, IL, USA) at the wavelength of 540 nm.

12. Invasion assay (II)

The upper chamber of a 24-well transwell (Corning™ Falcon™ Cell Culture Inserts, 8.0 µm pore size, Fisher Scientific) was covered with 50 µL of 2.5 mg/mL matrigel containing 5 µg/mL fibronectin. 600 µL of the RPMI 1640 medium (GIST882 and GIST48) supplemented with 20% fetal bovine serum or 600 µL of the DMEM medium (GIST-T1) supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin were added to the lower chamber of the transwell. For the assay, 30,000 GIST48 cells, 20,000 GIST882 cells, and 10,000 GIST-T1 cells were plated in a medium without serum to the upper chamber of the transwell. GIST48 and GIST882 cells were incubated for 24 hours and GIST-T1 cells for 96 hours. Cell invasion was measured in 3 separate experiments by counting the cells within 10 photographed fields of the microscope (Leica CTR6000, Leica microsystems, Bannockburn, IL, USA; magnification ×200). The number of invaded cells was expressed as the average number of invaded cells per one microscope field.

13. Western blotting (I, II, III)

Cultured GIST cell lines were rinsed in PBS (Lonza, Walkersville, MD, USA) and scraped on ice into a RIPA lysis buffer (Pierce Biotechnology, Rockford, IL, USA) containing a Pierce Protease and Phosphatase Inhibitor Tablet (Thermo Fisher Scientific Inc., Rockford, IL, USA), followed by sonication. The protein concentrations were measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc.). Ten µg of protein was separated using gel electrophoresis and blotted onto an Immobilon-P Transfer Membrane (Millipore, Billerica, MA, USA). The primary antibodies and dilutions used are represented in the Table 3. The peroxidase-conjugated AffiniPure Goat Anti-Rabbit antibody was used as the secondary antibody (dilution 1:10,000; Jackson Immuno Research, West Grove, PA, USA). Blot immunostains were treated with a SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA), exposed to an X-ray film, and the films were developed with a Kodak Medical X-ray Processor 102 (Eastman Kodak, Rochester, NY, USA).

Table 3. List of antibodies used in Western blotting

Protein	Antibody	Origin and class	Dilution	Manufacturer
β-Actin	A300-491A	Rabbit, polyclonal	1:10000	Bethyl Laboratories
ITGA4	8440	Rabbit, monoclonal	1:1000	Cell signaling technologies
KIT	A4502	Rabbit, polyclonal	1:10000	DAKO
p-KIT (Y703)	3391	Rabbit, polyclonal	1:10000	Cell signaling technologies
PDE3A	HPA014492	Rabbit, polyclonal	1:1000	SIGMA
PDE3B	HPA024342	Rabbit, polyclonal	1:1000	SIGMA
SLFN12	ab113238	Rabbit, polyclonal	1:1000	Abcam
SLUG	9585	Rabbit, monoclonal	1:1000	Cell signaling technologies

14. mRNA and miRNA sequencing (III)

GIST882 and GIST48 cells were plated at a density of 900,000 cells/ on 6-well plates. After 24 hours, GIST cells were treated with 10 μM anagrelide. The cells were scraped into a lysis buffer at time points 0 h, 8 h, 24 h and 48 h and for miRNA sequencing at time points 0 h, 1 h, 8 h and 48 h. RNA extractions were done by NucleoSpin® RNA kit (Macherey-Nagel) and DNA extractions were done by QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA). Sequencing was performed at BGI (Shenzhen, China) using an Illumina HiSeq 2000 sequencer (Illumina, San Diego, CA, USA).

15. Mass spectrometry (III)

GIST882 and GIST48 cells were plated in the T-75 bottles, grown until 80 % confluence, and then treated with 10 μM of anagrelide hydrochloride for 24 h or 48 h, after which, cells from three replicate samples were scraped into an 8 M UREA. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed with an Orbitrap Elite hybrid mass spectrometer coupled to an EASY-nLC II system (Thermo Fisher Scientific). LC-MS/MS analysis, MS1 quantification and subsequent protein identification were performed as described in elsewhere (Loukovaara et al. 2015). For protein identification, the scan data acquired from LC-MS/MS were searched against the human component of the UniProtKB- database (release 2014_01) using SEQUEST search engine in Proteome Discoverer software (Thermo Fisher Scientific). Maximum one missed cleavage was allowed.

16. mRNA and miRNA sequencing data analysis (III)

SePIA (Sequence Processing, Integration, and Analysis) comprehensive transcriptomics workflow was used to quality control, pre-process and analyse the miRNA and mRNA data (Icay et al. 2016). Standard adapter and quality trimming (Bolger et al. 2014), read alignment (Dobin et al. 2013, Langmead et al. 2009) and quantification (Anders et al. 2015, Trapnell et al. 2012) were performed using SePIa. Normalization and differential analysis of miRNA expression between GIST882 and GIST48 at four times points was performed with the R package DESeq (Anders and Huber. 2010), a nominal *P* value of 0.01 was used. Normalized expression was used to identify significant, inversely correlated miRNA and mRNA expression ($P \leq 0.05$ and Pearson correlation coefficient ≤ -0.7). These miRNA-mRNA pairs were then cross-filtered with target prediction databases (Griffiths-Jones et al. 2008, Grimson et al. 2007, Kertesz et al. 2007, Maragkakis et al. 2009) so that a pair was supported by at least one database. Fold-change values were calculated for each of these miRNAs and target genes using average expression values in each cell line. Gene fold-change values were used as input for signal pathway impact analysis (SPIA (Tarca et al. 2009)). Genes and their predicted targeting miRNAs were then identified for each significant pathway (SPIA pGFDR ≤ 0.1).

17. GIST xenografts (III)

The GIST xenograft mouse models were generated in prof. Patrick Schöffski's laboratory (KU Leuven, Belgium). One model was based on the GIST882 cell line and three models were patient-derived. The GIST882 model (with *KIT* exon 13 mutation leading to p.K642E) and the UZLX-GIST3 model (with *KIT* exon 11 mutation leading to p.W557_V559delinsF) were considered imatinib-sensitive. The UZLX-GIST2B model was considered dose dependently imatinib-sensitive (with *KIT* exon 9 mutation leading to p.A502_Y503dup) and UZLX-GIST9 imatinib-resistant (with *KIT* exon 11 mutations leading to p.P577del and W557LfsX5, and a secondary *KIT* exon 17 mutation leading to p.D820G).

Adult female athymic Naval Medical Research Institute mice (NMRI nu/nu, Janvier Laboratories, Le Genest-Saint-Isle, France) were transplanted by subcutaneous inoculations of GIST882 cells or subcutaneous transplantation of biopsies on both sides bilaterally as described previously (Floris et al. 2009). Mice were 10-12 weeks old at the time of the transplantation and randomly assigned to different treatment groups. Two control mice cohorts were used, one treated with 10% ethanol

(vehicle for anagrelide, bid), and one with imatinib (100 mg/kg/qd). The test mice were treated with anagrelide (5 mg/kg/bid) or with the combination of anagrelide and imatinib. Effectiveness of the treatment in the anagrelide group was compared to the control groups. Each group consisted of 4 to 6 mice. The tumor volumes (measured 3 times per week) and the mice body weight (measured daily) was assessed for 10 to 29 days until the mice were sacrificed (or when the tumor diameter exceeded 2 cm, the loss of the bodyweight was >20 %, or for another ethical reason).

Both frozen tissue samples and FFPE tissue samples were collected from the sacrificed mice for molecular analyses (Table 4 and 5). The FFPE tumor specimens were cut to 4-µm sections for staining with haematoxylin and eosin (H&E) and for immunohistochemistry. The quantity of tumor necrosis, myxoid degeneration and/or fibrosis was evaluated on the H&E stained slides, and graded as either grade 1 (0 %–10 %), grade 2 (>10 % and ≤50 %), grade 3 (>50 % and ≤ 90%) or grade 4 (>90 %) as described elsewhere (Antonescu et al. 2005).

The patient-derived GIST model xenografting was approved by the Medical Ethics Committee, University Hospitals Leuven (Leuven, Belgium). The *in vivo* experiments were approved by the Ethics Committee for Animal Research, KU Leuven (Leuven, Belgium) and were conducted according to their guidelines and Belgian regulations.

Table 4. List of antibodies used in xenograft immunohistochemistry.

Protein	Antibody	Dilution	Incubation time	Pre-treatment buffer	Manufacturer
Cleaved PARP	Ab32064	Ready to use	1 h at RT	Reveal decloaker + water bath	Abcam
DOG1	NCL-L-DOG-1	1:250	30 min at RT	EDTA + water bath	Novocastra
Ki-67	RM9106	Ready to use	30 min at RT	Sodium citrate + water bath	Thermo scientific
p-AKT	4060	1:25	Overnight at +4°C	Sodium citrate + water bath	Cell signaling technologies
p-Histone H3	9701	1:100	Overnight at +4°C	Sodium citrate + water bath	Cell signaling technologies

Table 5. List of antibodies used in xenograft Western blotting.

Protein	Antibody	Origin and class	Dilution	Manufacturer
AKT	9272	Rabbit, polyclonal	1:1000	Cell signaling technologies
pAKT (S473)	9271	Rabbit, polyclonal	1:1000	Cell signaling technologies
Histone H3	4499	Rabbit, monoclonal	1:1000	Cell signaling technologies
pHistone H3	9701	Rabbit, polyclonal	1:1000	Cell signaling technologies
KIT	A4502	Rabbit, polyclonal	1:5000	DAKO
pKIT (Y719)	3391	Rabbit, polyclonal	1:1000	Cell signaling technologies
pKIT (Y703)	3073	Rabbit, polyclonal	1:1000	Cell signaling technologies
MAPK	9102	Rabbit, polyclonal	1:1000	Cell signaling technologies
pMAPK	4370	Rabbit, monoclonal	1:1000	Cell signaling technologies
Tubulin	2128	Rabbit, monoclonal	1:1000	Cell signaling technologies

18. Statistical analysis (I, II, III)

The frequency tables were analysed with the chi^2 test or Fisher's exact test. The Kaplan-Meier method, the univariable Cox proportional hazards model, and the log rank test were used to estimate the cumulative survival. Overall survival was calculated from the date of the GIST diagnosis to the date of death, censoring patients who were alive on the last date of follow-up. GIST-specific survival was computed from the date of the diagnosis to death deemed to result from GIST, censoring patients who died from another cause on the date of death and patients who were alive on the last date of follow-up. Recurrence-free survival (RFS) was calculated from the date of randomization to the date of GIST recurrence or to death, whenever death preceded recurrence, censoring the patients alive on the date of last follow-up. The interaction between tumor SLUG expression and imatinib treatment in the SSGXVIII/AIO series (study I) was calculated using a Gaussian process model for conditional event probabilities (Joensuu et al. 2012). The Mann-Whitney test or the Kruskal-Wallis test was used to compare the continuous parameters. Comparisons between the groups in the TUNEL analysis, proliferation analysis, invasion analysis, cAMP and cGMP analyses, histopathologic results and tumor volume between different mice treatment groups were done using the Mann–Whitney U-test. The Wilcoxon matched paired test was used for tumor volume comparisons between day 1 and at the end of every *in vivo* experiment. All *P* values are 2-sided. The statistical calculations were done using the IBM SPSS Statistics package v. 22.0 (IBM, Armonk, NY, USA), STATISTICA 13.0 (Dell Statistica, Tulsa, OK, USA) or GPstuff 4.6 (Vanhatalo et al. 2013).

RESULTS

1. SLUG, ITGA4, PDE3 expression, clinical factors and disease outcome (I, II, III)

We investigated the MediSapiens *in silico* database of human transcriptomes for genes that are highly expressed in GISTs, and those with protein products known as drug targets, to identify potential driver oncogenes in GIST. The outlier statistics was performed with the gene-tissue index (GTI) analysis. *ITGA4*, *PDE3A* and *PDE3B* mRNA expressions were high in GISTs as compared with other types of cancer and healthy gastrointestinal tract tissues. Similarly, the analysis identified genes that are well known to be overexpressed in GIST such as *KIT*, *anoctamin 1 (ANO1)* and *protein kinase C theta* (*PRKCQ*). *PDE3A* and *PDE3B* mRNAs were expressed particularly in GIST.

The expression of SLUG, ITGA4, PDE3A and PDE3B were studied in three different clinical cancer patient series and analyzed by immunohistochemistry. SLUG expression was present 125 (25.0 %) out of 500 GISTs, ITGA4 expression 106 (52.2 %) out of 203 GISTs, PDE3A was expressed in 50 (90.9 %) of the 55 GISTs investigated, and PDE3B in 33 (60.0 %). ITGA4, PDE3A and PDE3B expression was also detected in other cancer types like leiomyosarcoma, another common type of abdominal sarcoma, but much less frequently than in GIST. SLUG expression was studied only in GIST sample series but SLUG expression has been described in a wide spectrum of human cancers (Cobaleda et al. 2007). In Studies II and III the the specificity of immunostaining was examined by comparing the protein expression at immunohistochemistry with mRNA expression measured with qPCR ($P \leq 0.001$ in both studies).

The clinical significance of protein expressions was investigated in two series of GIST patients (the Western Sweden study and the SSGXVIII/AIO trial for high risk patients). In the Western Sweden series SLUG and ITGA4 were associated with presence of tumor necrosis, a high tumor mitotic count and presence of metastases at the time of GIST detection. In addition, SLUG was associated with a large tumor size at the time of the diagnosis, epithelioid type of tumor histology and nuclear pleomorphism; and ITGA4 with a tumor location in the stomach, a high NIH risk stratification group for recurrence (all P values < 0.05). In the SSGXVIII/AIO study tumor SLUG expression was significantly associated only with the presence of tumor rupture in high risk patients.

Patients whose GIST expressed SLUG had less favorable overall and recurrence free survival (RFS) than patients whose GIST did not express SLUG ($P = 0.006$ and $P = 0.001$, respectively). Whereas ITGA4 expression was associated with poor overall survival and GIST-specific survival ($P = 0.044$ and $P = 0.008$, respectively). The associations of clinical factors with PDE3A and PDE3B protein expression was also studied in the population-based Western Sweden series, but no significant correlations for clinicopathological factors and RFS or overall survival were found ($P > 0.05$, unpublished data). In the SSGXVIII/AIO trial, high risk patients with SLUG-positive tumor had inferior RFS as compared to patients whose GIST did not express SLUG ($P = 0.001$).

2. Effect of SLUG or ITGA4 downregulation on GIST cell lines (I, II)

As SLUG and ITGA4 expressions were associated with various clinical high-risk features and unfavorable survival, the function of these proteins was further investigated in the GIST cell lines. The function of SLUG was investigated in GIST48 and GIST882 cell lines. Additionally, ITGA4 expression was studied in GIST-T1 cell line. Both SLUG and ITGA4 were present in the GIST cell lines. SLUG was, however, expressed substantially more weakly in GIST882 as compared with GIST48.

Both tumor SLUG and ITGA4 expressions were associated with a high GIST cell proliferation rate in the Western Sweden series, and we investigated whether SLUG or ITGA4 expression promote GIST cell viability using the MTT assay. SLUG RNA knockdown reduced cell proliferation in GIST48 and GIST882 cell lines ($P < 0.001$ for each cell line). In addition, downregulation of SLUG increased cell death in these cell lines when the numbers of dying cells were measured using the TUNEL assay. In the GIST48 cell line, the mean proportion of dying cells increased from 10.7% (SE = 1.8%) in the control to 28.4% (SE = ±4.2%, $P = 0.008$) after treating the cells with SLUG siRNA. Similarly, in the GIST882 cell line SLUG siRNA transfection increased the mean proportion of DNA fragmentation in nuclei from 18.3% (SE = ±2.6%) in the control siRNA to 33.2% (SE = ±5.1%, $P = 0.029$). In GIST882 cell line, downregulation of SLUG showed synergy with imatinib and sensitized cells to imatinib-induced apoptosis.

Blocking of ITGA4 by RNA interference had no effect on the proliferation of the 3 GIST cell lines (all P-values > 0.05). VLA-4 (α4β1) inhibition by BIO1211 and BIO5192 had only little effect on proliferation. The effect of ITGA4 downregulation and VLA-4 inhibition for GIST cell invasion was

further evaluated in a matrigel-coated transwell system. The number of invaded cells decreased in all GIST cell lines after ITGA4 siRNA transfection as compared with the control siRNAs (GIST48, mean = 8.3 vs. control mean = 23.2, $P = 0.016$; GIST882, mean = 3.4 vs. control mean = 29.7, $P = 0.014$; and GIST-T1, mean = 1.5 vs. control mean = 17.4, $P < 0.001$). Similarly, the number of invaded cells decreased in each GIST cell line after VLA-4 inhibitor treatments. However, in GIST882 and GIST-T1 cell lines, only BIO1211 decreased the numbers of invaded cells significantly.

3. Effect of PDE3 inhibition on GIST cell proliferation and signaling (III)

GIST48 and GIST882 cell lines were first tested for sensitivity to 217 drug compounds. The drug response profiles were compared with the expression of the top 25 genes that were highly expressed on GIST based on the GTI-analysis. Various tyrosine kinase inhibitors including axitinib, dasatinib, foretinib, imatinib, nilotinib, ponatinib, sorafenib, sunitinib and tivozanib reduced the viability of GIST cells. Unexpectedly, a phosphodiesterase 3 inhibitor, anagrelide, which is marketed for the treatment of patients with thrombocytemia, was also effective on the GIST882 cells.

The effect of anagrelide and three other PDE3 inhibitors were next investigated closer in the GIST48 and GIST882 cell lines. As expected, anagrelide induced a strong cytotoxic effect on GIST882 cell line ((IC50=0.016 μM). The efficacy of anagrelide was comparable to the effect of imatinib. However, the anagrelide was only weakly active in the GIST48 cell line. The anagrelide plus imatinib combination decreased the proliferation of GIST cells substantially compared to single agent treatments. Amrinone, cilostazol and milrinone had no effect on GIST48 or GIST882 cell proliferation.

We investigated further the effect of anagrelide and the combination of anagrelide and imatinib in GIST cells with TUNEL assay that detects the fragmented DNA and the proportion of apoptotic cells. 21.7 % of the anagrelide-treated GIST882 cells were apoptotic, while only slight DNA fragmentation was seen in GIST48 cell line. In GIST882 cells treated with imatinib, the proportion of apoptotic cells was comparable to anagrelide-treated cells. In GIST48, cells imatinib increased cell death to 32.1 % as compared to control. In addition, a larger proportion of the cells entered apoptosis in both GIST cell lines when imatinib and anagrelide were co-administered. 36.8 % of GIST882 cells and 44.1 % of GIST48 cells were apoptotic after the combination treatment.

The effect of four PDE3 inhibitors on the PDE3 activity were analyzed by measuring changes in the intracellular cAMP and cGMP levels in the GIST cell lines. An increase in the intracellular cAMP and cGMP in response to PDE3 inhibition was observed in both GIST cell lines. Of the compounds tested, anagrelide inhibited the PDE3 enzyme activity most effectively. Increase of cAMP was greater in GIST882 cells, where all four PDE3 inhibitors induced a significant increase ($P < 0.05$), while only milrinone and anagrelide induced significant increase in GIST48 cells. Similarly, the cGMP level changes were more remarkable in GIST882 cells. In GIST882 cells milrinone and anagrelide and in GIST48 cells anagrelide increased the cGMP levels significantly.

The role of PDE3A and PDE3B in GIST cells were investigated by blocking their expression using small interfering RNAs. The inhibition of PDE3A did not influence GIST cell proliferation. PDE3B downregulation decreased the proliferation of the GIST882 cell line ($P < 0.01$), whereas only weak decrease of proliferation was seen in the GIST48 cell line ($P < 0.05$).

In addition, we treated GIST882 cells with a combination of PDE3 siRNAs and increasing concentrations of anagrelide. PDE3B siRNA and the combination PDE3A siRNA and PDE3B siRNA increased the cytotoxic effect of anagrelide, whereas PDE3A siRNA decreased the sensitivity to anagrelide. None of the treatments influenced GIST48 viability. GIST cells were treated also with 0.5 μM anagrelide and rising concentrations (from 0.0001 μM to 30 μM) of PDE inhibitors cilostazol, milrinone or amrinone for 72 hours. Cilostazol and milrinone rescued cell death induced by anagrelide in GIST882 cells, whereas amrinone had no effect, and none of the treatments had an effect on the viability of GIST48 cells.

Downregulation of KIT or PDE3B with siRNAs increased apoptosis in both cell lines. In the GIST48 cell line, the mean proportion of dying cells increased from 10.7% in the control to 27.4% ($P = 0.078$) after treating the cells with PDE3B siRNA and to 34.7 % ($P < 0.001$) after treating the cells with KIT siRNA. In the GIST882 cell line PDE3B and KIT siRNA transfections increased the mean proportion of DNA fragmentation from 18.3% in the control siRNA to 57.3 % ($P < 0.001$) and to 35.4 % ($P = 0.045$), respectively. PDE3A or PDE3B knockdown had no effect on KIT or phospho-KIT expression on GIST cell lines. Indicating that PDE3 signaling is may not be directly linked to KIT signaling. In previous a study of de Waal et al. speculated that anagrelide might promote the interaction between PDE3A and SLFN12 (de Waal et al. 2016). We studied if SFLN12 is expressed in GIST cells, but found no SLFN12 expression.

GIST cell line mRNA and miRNA expressions were studied using RNA-sequencing to identify the essential signaling pathways that were affected by anagrelide treatment, and the protein expression was investigated using label-free liquid chromatography-mass spectrometry. Comprehensive transcriptomic data analysis and integration workflow, SePIA, was used to identify genes whose expression patterns were inversely correlated with miRNA expression patterns and accumulated perturbations in the corresponding signaling pathways. The pathway perturbations were compared between the GIST882 cell line (anagrelide sensitive) and in GIST48 cell line (anagrelide insensitive), and the most remarkable changes were found within the pathways such as cell cycle, focal adhesion, oocyte meiosis, protein processing in the endoplasmic reticulum, insulin signaling, p53 signaling, TGF-beta signaling, Wnt signaling and neurotrophin signaling. The protein expression of the cell lines was next correlated with the significantly regulated and enriched genes in the perturbation pathways. Fifteen proteins including YWHAQ, PPP1CB, EIF4E, CALM2 and RAD23B showed complete overlap with mRNA expression and an inverse correlation with miRNA expression.

4. PDE3 inhibition in GIST xenograft mouse models (III)

We studied the *in vivo* efficacy of anagrelide in a GIST822 cell line xenograft model and in three patient-derived GIST xenograft mouse models. The tumor growth inhibition or size reduction was found in three out of the four models. The most potent response was seen in GIST2B that harbors the *KIT* exon 9 p.A502_Y503dup duplication mutation, where anagrelide caused a marked tumor volume regression (68%) after 10 days of treatment and was substantially more effective than imatinib. Evaluation of the histologic response (HR) showed that anagrelide led to the induction of grade 2 or higher HR in 50 % of the GIST2B tumors. The imatinib plus anagrelide combination treatment led to a superior HR compared to single agent treatments.

In the GIST882 xenograft model, imatinib or anagrelide single agent treatments stabilized the tumor volume, while their combination treatment resulted in a substantial reduction in the tumor volume. Single-agent anagrelide caused a minimal HR (majority grade 1), but a superior HR was observed (grade 2/3 in most of the tumors) in the combination treatment group.

Single-agent anagrelide stabilized the tumor volume ($P = 0.09$) and led to a grade 2 HR (in 50 % of the tumors) in the GIST3 model with *KIT* exon 11 indel mutation (p.W557_V559delinsF). In this model imatinib was, however, more effective than anagrelide leading to significant tumor regression.

In addition, the effect of the combination of anagrelide plus imatinib on the tumor growth was similar to single agent imatinib treatment. However, a more marked HR was achieved with the anagrelide plus imatinib combination as compared the single agent treatments. None of the treatments tested was effective in the GIST9 model with *KIT* primary exon 11 mutation and a secondary *KIT* exon 17 mutation, although the tumors grew slightly slower in all treatment groups as compared to the control group.

The anti-proliferative and apoptotic activities of the different drug treatments was evaluated with the H&E staining and with immunohistochemistry for phospho-histone H3, Ki67 and cleaved PARP expression. The control tumors showed a high proliferative activity in all tumor models (an average of 40 mitotic cells per 10 high power fields). Anagrelide treatment reduced significantly the cell proliferation rate in the GIST2B and GIST3 models compared to the control groups ($P \leq 0.005$). A combination treatment with anagrelide and imatinib reduced significantly the mitotic activity compared to single-agent treatments in the GIST882 and GIST2B models. Anagrelide increased apoptosis in the GIST3 and GIST882 models compared to the control groups. In addition, the combination treatment induced a more potent apoptotic activity compared to imatinib or anagrelide alone in these two models (the mouse xenograft cell proliferation and apoptotic rate results are unpublished data).

The effect of anagrelide on KIT signaling and the effect of imatinib on PDE3 levels was evaluated by Western blotting. Anagrelide had no effect on KIT signaling in any of the GIST models. However, in GIST3, GIST2 and GIST882 models the combination treatment reduced tumor KIT and AKT levels, which may be caused by a higher HR (greater tumor necrosis and small cell viability) than the actual downregulation of these proteins. None of the treatments influenced markedly GIST PDE3A or PDE3B expression levels. GIST PDE3 expression levels showed no obvious association with response to anagrelide, but, interestingly, in the treatment-resistant GIST9 model the PDE3A to PDE3B ratio was the highest. We investigated also whether SLFN12 is expressed in the GIST xenograft mouse models. Interestingly, the SLFN12 expression increased in anagrelide treated tumor tissues compared to the control group.

DISCUSSION

Several multikinase inhibitors are highly effective in the treatment of the most patients with advanced GIST (Bauer and Joensuu. 2015), but secondary drug resistance to imatinib and other tyrosine kinase inhibitors usually eventually develops leading to GIST progression (Heinrich et al. 2006). The second and the third line treatments with the approved multikinase inhibitors sunitinib (Demetri et al. 2006) and regorafenib (Demetri et al. 2013) are effective, but the responses are often of short duration, and neither commercially available treatment is curative, causing an unmet clinical need in GIST therapy. Clinical studies and new therapy options are heavily focused on the receptor tyrosine kinase inhibitors. In the present thesis, we investigated the role of SLUG, ITGA4 and PDE3s as novel therapeutic targets for the treatment of GIST patients.

1. SLUG is a pro-survival and prognostic factor in GIST

The role of KIT receptor tyrosine kinase in GIST formation is well established, but the molecular mechanism beyond KIT signaling are still incompletely understood. The E-twenty-six (ETS) family transcription factor ETV1 is a regulator of the interstitial cells of Cajal (ICC) lineage and has a key role in the development of GIST (Chi et al. 2010). Further, it was found that the forkhead family member FOXF1 controls the transcription of two GIST master regulators, *KIT* and *ETV1* (Ran et al. 2017). We investigated whether the transcription factor SLUG, which has been linked with the KIT signaling pathway (Motro et al. 1991, Perez-Losada et al. 2002, Sanchez-Martin et al. 2003), also has a function in GIST tumorigenesis. The clinical significance of SLUG in GIST has not been reported previously. We found that SLUG is expressed in 25 % of GISTs and that the SLUG expression was associated with unfavorable RFS in two separate clinical series. In addition, SLUG was associated with several known risk factors for GIST recurrence and metastases such as presence of tumor rupture, a high tumor mitotic count and presence of metastases at the time of GIST detection. However, in the present study SLUG expression was not associated with non-gastric tumor location, which is considered an established adverse prognostic factor in GIST (Joensuu et al. 2016).

In line with our findings, patients with SLUG-positive cancers have poor clinical outcomes in several human cancer types such as breast cancer, colorectal cancer, lung cancer and esophageal cancer (Liu et al. 2013, Shioiri et al. 2006, Uchikado et al. 2005, Wu et al. 2015). Consequently, tumor SLUG expression is likely an adverse prognostic feature in various human cancers. We found that SLUG

expression in GIST is a relatively strong adverse factor in univariable survival analyses of two GIST patient series, and it had independent prognostic value in a trial including high risk patients treated with surgery and adjuvant imatinib. However, in our series most of even high-risk GISTs did not express SLUG proposing that elevated SLUG expression may not invariably be related to tumor progression in GIST.

SLUG downregulation decreased cell viability and increased cell death in prostate cancer and neuroblastoma (Emadi Baygi et al. 2010, Vitali et al. 2008). In leiomyosarcomas the knockdown of SLUG was associated with decreased cell proliferation and invasion (Yang et al. 2010). Interestingly, the combination of SLUG knockdown plus imatinib inhibited also the metastatic growth of neuroblastoma in an *in vivo* model, while imatinib as a single agent was unable to reduce the metastatic burden (Vitali et al. 2008). Similarly, we found that blocking of SLUG expression reduced GIST cell proliferation and increased apoptosis. Downregulation of SLUG showed synergy also with imatinib on GIST882 cell line proliferation and sensitized cells to imatinib-induced apoptosis. In ovarian cancer cells SLUG contributes to cisplatin resistance, and SLUG silencing increases the sensitivity of ovarian cancer to drug treatment (Haslehurst et al. 2012). It has been suggested that SLUG may prevent apoptosis either by regulation of caspases or repressing expression of the pro-apoptotic PUMA protein (Tribulo et al. 2004, Wu et al. 2005). SLUG has a central role in a signaling involved in survival and imatinib resistance in chronic myeloid leukemia progenitor cells (Mancini et al. 2010). Similar mechanisms may also contribute drug resistance in GIST, and SLUG might contribute to imatinib resistance in GIST. SLUG may mediate cell survival and drug resistance in cancer cells by regulation set of genes functioning in various signaling pathways. Transcription factors ETV1 and FOXF1 have been described as a potential new therapy targets in GIST (Chi et al. 2010, Ran et al. 2015, Ran et al. 2017). Theoretically, imatinib and SLUG down-regulation could be combined for the treatment of some malignancies including GIST, but the means to deliver effective anti-SLUG therapy efficiently and safely are currently lacking. However, novel technologies such as proteolysis targeting chimeras (PROTACs) that use bifunctional small molecules to link the target proteins to the E3 ubiquitin ligase system for protein degradation may provide a new option for the therapeutic targeting of transcription factors (Deshaies. 2015).

The molecular mechanisms how KIT signaling may regulate SLUG are controversial. In colorectal cancer cell lines KIT expression suppressed SLUG (Gavert et al. 2013), but KIT increased SLUG expression and KIT downregulation decreased SLUG expression in malignant mesothelioma and

neuroblastoma cells, whereas upregulation of KIT increased SLUG expression in leukemia cells (Catalano et al. 2004, Lau et al. 2015, Perez-Losada et al. 2002). Taken together, SLUG signaling is likely influenced also by other factors than KIT, and this may occur in a tumor phenotype-dependent manner. A recent study shows that the forkhead box (FOX) family transcription factor FOXF1 controls the transcription of two key regulators, *KIT* and *ETV1*, both likely frequently required for GIST tumorigenesis (Ran et al. 2017). As a potential core-controller of the GIST pathogenesis, FOXF1 may influence also on SLUG signaling.

2. Integrin alpha 4 (ITGA4) contributes to GIST invasion

Relatively little is known about the molecular factors that influence the invasive and metastatic properties of GIST. Integrins mediate a wide variety of cellular effects and signalling that can result in tumor progression and metastasis in various types of human cancer (Desgrosellier and Cheresh. 2010) but their significance in GIST is poorly understood. It is not unexpected that integrins may be important also in the molecular pathogenesis of GIST. At present, the role of ITGA4 is unknown in GIST.

ITGA4 was expressed in over 50 % of GISTs, and the expression was associated with high mitotic counts, tumor necrosis, poor survival and a presence of distant metastases during the course of disease. These observations are in line with the earlier reported role of α4 integrins in the embryonal development, normal tissues, and in cancer. The alpha 4 integrins are expressed especially during hematopoiesis on early pluripotent progenitor cells, eosinophils, and mononuclear leukocytes (Arroyo et al. 1999). The alpha 4 integrins mediate cell-cell adhesions that may be important also for the immune functions (Kinashi. 2012). They have an important role in leukocyte trafficking, and they may be involved in the pathogenesis of several chronic diseases including multiple sclerosis, rheumatoid arthritis, contact hypersensitivity, and inflammatory bowel diseases (Abraham et al. 1994, Chisholm et al. 1993, Yednock et al. 1992). Natalizumab, a recombinant humanized monoclonal ITGA4 antibody, has demonstrated clinical activity in patients with relapsing multiple sclerosis and Crohn's disease (Ransohoff. 2007, Rutgeerts et al. 2009).

Although ITGA4 is known to influence especially cell migration and invasion, it may also have an effect on cell proliferation. Activation of ITGA4 leads to tumor inflammation and the growth of myeloid cells (Schmid et al. 2013), whereas, natalizumab may decrease multiple myeloma

progression by blocking the interaction between multiple myeloma cells and the bone marrow stromal cells (Podar et al. 2011). In our clinical GIST patient series ITGA4 expression was strongly associated with a high tumor mitotic count. However, the knockdown of ITGA4 or the inhibition of VLA-4 had no effect on the GIST cell proliferation. ITGA4 expression is associated with poor prognosis especially in chronic lymphocytic leukemia, but also in neuroblastoma (Buggins et al. 2011, Bulian et al. 2014, Young et al. 2015). In agreement with these observations, tumor ITGA4 expression was associated with with poor overall and GIST-specific survival in our GIST patient series. ITGA4 and VCAM-1 interaction increases the metastatic capacity of beta2/beta3-negative melanoma cells (Klemke et al. 2007), and downregulation of VLA-4 (α4β1) in a MV3 melanoma *in vivo* model with heparin derivatives reduces melanoma metastatic potential (Schlesinger et al. 2012). We found a positive association between GIST ITGA4 expression and metastatic disease in a GIST patient series, and downregulation of *ITGA4* mRNA expression or VLA-4 inhibition in GIST cell lines decreased the invasion of GIST cells. The role of ITGA4 in GIST progression relate more likely with the contribution of ITGA4 to tumor invasion and dissemination rather than to cell proliferation.

It has been suggested that ITGA4 has a role in cancer drug resistance in leukemia. ITGA4 was identified as a central mediator of drug resistance of pre-B-cell acute lymphoblastic leukemia, and blocking of ITGA4 sensitized leukemia cells to chemotherapy (Hsieh et al. 2013). ITGA4-fibronectin interactions on acute myeloid leukemia cells reduce their chemosensitivity (Matsunaga et al. 2003). In small cell lung cancer ITGA4 signaling mediates adhesion, survival and chemoresistance (Hartmann et al. 2005). Further, the ITGA4 - Abi-1 (Abelson interactor-1) signaling pathway and the high expression levels of ITGA4 may mediate the imatinib drug-resistance in Bcr-Abl-positive leukemia (Chorzalska et al. 2014). Abl has structural similarity with KIT that makes this observation especially interesting.

The focal adhesion kinase (FAK), one of the key mediators of integrin signaling and a promoter of cell migration, is highly expressed in GISTs (Koon et al. 2004, Mitra and Schlaepfer. 2006). Somewhat unexpectedly, imatinib was found to stimulate the SRC family kinase (SFK) and FAK activation, and thus integrin signaling in a mouse $Kit^{V558\Delta/+}$ model of GIST in one study (Rossi et al. 2010) suggesting that integrin signaling may be important for tumor persistence and emergence of imatinib resistance. In addition, integrins αvβ1, αvβ3, αvβ5 and their ligand, the neuronal adhesion protein L1CAM, are expressed in GIST (Kaifi et al. 2006, Rossi et al. 2010). In accordance with earlier results (Rossi et al. 2010), imatinib treatment induced FAK Y397 phosphorylation in GIST48

and GIST-T1 cell lines (unpublished data). Both the basal level and imatinib-induced phosphorylation of FAK decreased markedly in GIST cell lines after suppression of *ITGA4* mRNA, suggesting that ITGA4 has a role in the induction of FAK phosphorylation and migration of GIST cells.

Regardless of the encouraging preclinical results, clinical trials using integrin inhibitors have been mostly disappointing (Desgrosellier and Cheresh. 2010). The complexity of integrin function and integrin-GFR interaction makes integrins difficult to target, and signaling molecules downstream of integrins might be easier to target therapeutically. Several clinical phase 1-2 trials are currently ongoing for example with FAK and Src inhibitors, but the therapeutic potential of these drugs is still uncertain.

3. PDE3 family is a potential therapeutic target in GIST

Phosphodiesterases are a useful drug target in a few diseases, and they might be a therapeutic target in the treatment of some cancers. In the present thesis study, we found that phosphodiesterases have a role also in the pathogenesis of GIST and that a phosphodiesterase 3 inhibitor, anagrelide, marketed for the treatment of patients with thrombocytemia, might be an active agent in the treatment of GIST.

We found that PDE3A and PDE3B enzymes are expressed especially highly in GIST as compared with other human tumors and histologically normal tissues, and propose that PDE3 activity might be important especially for GIST viability. Previously, PDE3A was found to be expressed in the gastric antrum and in the interstitial cells of Cajal in a GIST murine model harboring a germline *KIT* mutation (K641E) (Gromova et al. 2009). Overexpression of several other phosphodiesterases and reduced cAMP or cGMP expression have been found in colorectal cancer, glioblastomas, lung cancer and leukemias (Marko et al. 2000, McEwan et al. 2007, Zhang et al. 2008). Several PDE inhibitors have also been reported to induce apoptosis or cell cycle arrest in various cancer cell lines, such as breast cancer, melanoma and leukemia lines (Marko et al. 2000, Piazza et al. 2001, Saravani et al. 2012, Shimizu et al. 2009, Zhang et al. 2008). Interestingly, PDE3A modulator 6-(4-(diethylamino)-3-nitrophenyl)-5-methyl-4,5-dihydropyridazin-3(2H)-one (DNMDP) may decrease the viability of GIST cells (Vandenberghe et al. 2017). Vandenberghe et al. proposed also that PDE3 inhibitor, cilostazol, might reduce the viability of GIST cells. We were not able to replicate these results in our cell lines, and observed no effect on GIST cell proliferation after cilostazol treatment. This is in line with the observation that cilostazol does not replicate the cytotoxic effect of DNMDP in DNMDP-

sensitive cell lines (de Waal et al. 2016). Of the 11 different PDE families, the PDE3 and PDE4 families are the key families in the hydrolysis of the cAMP phosphodiester bonds (Conti and Beavo. 2007). All the PDE3 inhibitors tested had some effect on the AMP and/or cGMP levels in GIST cell lines, but anagrelide inhibited the PDE3 activity most effectively.

The role of PDE3s were investigated further by inhibiting the expression of PDE3A and PDE3B in GIST cell lines using RNA interference. PDE3B inhibition decreased the proliferation of GIST cells, while PDE3A reduction had no effect. The reduction was, however, weaker compared to KIT knockdown. The cytotoxic effect of anagrelide may not be caused only by direct inhibition of PDE3A or PDE3B enzyme activity. Interestingly, we observed that PDE3A knockdown decreased sensitivity to anagrelide, whereas PDE3B knockdown increased anagrelide-induced cytotoxicity. PDE inhibitors cilostazol and milrinone rescued anagrelide cytotoxicity suggesting that some PDE3 inhibitors may compete with anagrelide for binding to the same molecular target. Anagrelide cytotoxicity requires PDE3, and the cytotoxic effect of anagrelide may be mediated through modulation of the PDE3 signalosome complex that is dependent on both PDE3A and PDE3B.

Cyclic nucleotide signaling is organized via the formation of cyclic nucleotide signalosomes, which are localized macromolecular complexes that contain precisely recruited cyclic nucleotide effectors (Maurice et al. 2014). Disruption and interference of these complexes or displacing individual complex partners may lead to disturbed function of the signalosome. Some PDE3 inhibitors function as competitive inhibitors and occupy the catalytic cAMP or cGMP binding sites (Card et al. 2004, Zhang et al. 2002). The binding of DNMDP, a PDE3 modulator, to PDE3A promotes the interaction between PDE3A and Schlafen 12 (SLFN12) that mediates the death of Hela cells, whereas depletion of PDE3A rescues the cells from death (de Waal et al. 2016). We found that that anagrelide may increase the SLFN12 expression in GIST xenograft mouse tissues, but SLFN12 expression is probably not required to induce the cytotoxic effect of anagrelide in GIST cells. We found that a 14-3-3 family protein YWHAQ and protein phosphatase 1 subunit PPP1CB, known partners of the signalosomes of PDE3s (Maurice et al. 2014), were dysregulated at both gene and protein expression levels after the PDE3 inhibitor anagrelide treatment in the GIST cell lines.

Phosphodiesterases have been studied as potential anticancer targets in preclinical models (Savai et al. 2010), but so far no PDE inhibitor has been approved for the treatment of cancer. Most of the anticancer studies have focused on the PDE4 and PDE5 families. Phosphodiesterase inhibition has

shown antitumor efficacy especially in hematological malignancy models, in particular those of chronic lymphocytic leukemia and acute lymphoblastic leukemia (Lerner and Epstein. 2006, Ogawa et al. 2002). The PDE4 inhibitor rolipram attenuated cell proliferation and angiogenesis in a lung cancer xenograft mouse model, and reduced tumor growth and enhanced the effects of radiation therapy and chemotherapy in gliomas (Goldhoff et al. 2008, Pullamsetti et al. 2013). PDE4D inhibitors NVP-ABE171 and cilomilast decreased the growth of prostate cancer cells *in vivo* (Powers et al. 2015). PDE5 inhibitors in combination with chemotherapeutic agents could prevent progression and metastasis (Liu et al. 2016), and the PDE5 inhibitor sildenafil induced cell growth inhibition, cell cycle arrest and apoptosis of colorectal cancer xenograft mouse models (Mei et al. 2015).

Finally, we investigated the *in vivo* efficacy of the PDE3 inhibitor, anagrelide, in four GIST xenograft mouse models carrying diverse *KIT* mutations. The tumor volume reduction was observed in one model and tumor growth inhibition in two of the models. The most potent effect was observed in a GIST xenograft mouse model with *KIT* exon 9 mutation, which mutations occur in about 10% of all GISTs. Patients with *KIT* exon 9 mutations are also associated with inferior recurrence-free survival outcomes as compared with other *KIT* mutation types (Joensuu et al. 2015). GISTs carrying exon 9 mutation usually pose a therapeutic challenge, as these GISTs require a high daily dose of imatinib (800 mg/day), which often causes considerable adverse effects (Gastrointestinal Stromal Tumor Meta-Analysis Group (MetaGIST). 2010). In GIST882 and GIST3 models, single-agent anagrelide treatment stabilized the tumor volume, and the combination of anagrelide and imatinib showed a synergistic effect on tumor viability.

Several PDE inhibitors have shown promising preclinical results especially when combined to some of the current treatment options. This could be the best approach also in the treatment of GIST. Results from a series of patient with chronic myeloproliferative disorders treated with the combination of imatinib and anagrelide suggest that their co-administration is feasible in the clinic (Tsimberidou et al. 2003). Based on our results, a clinical trial evaluating the effect of anagrelide in the treatment of advanced imatinib-resistant GIST seems highly warranted.

4. Study limitations

The quantitation of protein expression based on immunohistochemistry is subjective and many of the antibodies used were polyclonal, increasing the possibility of unspecific antibody binding. The type

and quality of tumor tissue used in the study may also have influenced the results, and we cannot exclude the effect of factors such as differences in tissue sample fixation. However, the immunohistochemistry scoring correlated with tumor mRNA qPCR measurements in a subset of cases ($P < 0.05$, II, III). In addition, the immunohistochemical scorings were compared between two independent observers and the agreement turned out to be good. In part, the studies were based on a subset of the patients. Therefore, we compared age at the time of the diagnosis, gender, the NIH risk classification distribution, and the main GIST prognostic features in the subset available for study and the entire population-based Western Sweden series. We did not find significant differences between the included subset and the entire series in any of these parameters, suggesting that patient exclusion caused no major bias. Yet, confirmation of the findings in other GIST series is warranted.

GIST is a relatively rare cancer type, like all sarcomas, and there are only few GIST cell lines available, and we had the access only to three of these. The findings from the studied GIST lines were, however, generally supportive to the data from the clinical series. The effects of siRNAs are often of relatively short duration, and the inhibition is rarely perfect. However, the siRNAs used turned out to work relatively well, and there was no remarkable variation between repeat experiments. Even though we observed that anagrelide is likely to modulate the action of PDE3, we were not able to show the exact mechanism of action of anagrelide.

5. Future studies

Outstanding advances have been achieved in the management of GIST over the last 20 years. Surgery remains the standard treatment for localized GIST, and with or without adjuvant imatinib, is considered the only curative treatment of GIST. Although imatinib and other tyrosine kinase inhibitors have remarkably improved the patient prognoses, drug resistance often eventually develops, and, therefore, new drug treatments are needed. In this thesis study, we investigated a few proteins that may have potential to function as therapeutic targets in the treatment of GIST.

Further investigation of the studied proteins and especially the effect and possible relationships of these proteins, and the ETV1 and FOXF1 transcription factors, might be beneficial. Studies on SLUG and ITGA4 in GIST xenograft mouse models might provide further information about the function of these proteins. Finding out the exact mode of action of anagrelide would probably be highly

valuable. Anagrelide is an old drug, and the co-administration of anagrelide and imatinib is probably feasible in the clinic. Therefore, the testing of anagrelide in a clinical trial is highly warranted.

The gene-tissue index analysis, which ranked the 25 best mRNA expression outlier genes for GIST, includes a few potential genes such as *PLAT*, *BCHE* and *NPR3* that could be targeted therapeutically, and are worth of further studies.

CONCLUSIONS

The aim of this thesis study was to find novel potential therapeutic drug targets for GIST treatment. We studied the role of SLUG, ITGA4 and PDE3 in GIST. The frequency of these proteins and their association with patient and tumor characteristics and disease outcome was studied with immunohistochemistry, and the functional role of the proteins was further investigated in GIST cell lines by inhibiting their function with siRNAs or protein specific inhibitors. The role of PDE3 was studied in patient-derived xenograft mouse models.

The main conclusions are the following:

1. SLUG expression was found in about a quarter of GISTs. SLUG expression was associated with unfavorable recurrence-free survival of GIST patients. SLUG may function as a pro-survival factor in GIST.

2. ITGA4 is highly expressed in GISTs compared to many other human cancers and histopathologically normal tissues. The ITGA4 protein expression is associated with several factors of unfavorable prognosis and the presence of metastases, and with poor GIST-specific and overall survival of the patients. ITGA4 may promote invasiveness and influence the clinical behavior of GIST.

3. PDE3A and PDE3B are frequently highly expressed in GISTs. Modulation of PDE3 activity decreased GIST proliferation and promoted apoptosis *in vitro*. A PDE3 inhibitor, anagrelide, reduced cell growth in patient-derived xenograft mouse models. Further testing of anagrelide in clinical trials is warranted.

The molecular mechanisms of GIST are still incompletely understood. This study reveals the role and function of three new potential molecular factors that may affect the function of the GIST cells and the prognosis of GIST patients. Further studies may show whether these proteins are of clinical importance, and will hopefully open up new therapy options for GIST patients.

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